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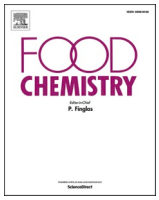

Comprehensive characterization of walnut oil processing by-products: biochemical composition, bioactive properties, and polyphenol *in vitro* bioaccessibility and bioavailability

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ABSTRACT

Underutilized by-products from the walnut-oil industry, namely walnut oilcake (WOC) and walnut oil dregs (WOD), were evaluated for their nutritional composition, phenolic compound profile and digestive behaviour, as well as bioactive properties (antioxidant, antimicrobial, anti-inflammatory, cytotoxic and prebiotic activities). WOC was rich in protein (38.1 g/100 g) and dietary fiber (32.6 g/100 g), while WOD presented high fat (46.8 g/100 g) and carbohydrate content (20.9 g/100 g). Glansreginin A was the predominant phenolic compound in both matrices. Following *in vitro* digestion using the INFOGEST protocol, higher overall polyphenol bioaccessibility was noticed in WOD (78%) compared to WOC (15%). Bioaccessible fractions exhibited higher antioxidant activity than the undigested samples. Glansreginin A was detected only on the cellular apical compartment suggesting the absence of transport across Caco-2 cells. After *in vitro* digestion, the non-bioaccessible fractions enhanced the growth of *Lactobacillus* and *Bifidobacterium* strains, in some cases surpassing fructooligosaccharides, a standard prebiotic. These findings support the valorisation of walnut by-products as functional ingredients, also contributing to sustainable food systems.

1. Introduction

The increasing consumption and variety of nut products are associated with the growing interest of consumers in foods that can confer beneficial health effects. In particular, walnut consumption has been associated with improvements in the blood lipid profile and a reduced risk of cardiovascular disease. (Song et al., 2022) recently provided a comprehensive overview of the bioactivities of walnut oil, highlighting its antihyperlipidemic, antioxidant, anticancer, anti-inflammatory, and antimicrobial properties, as well as its neuroprotective activity in preventing cognitive impairment.

Walnuts (*Juglans regia* L.) thrive in temperate climates worldwide, with an estimated annual production (kernel only) of 35 million tones. They are consumed in various ways, such as raw, in traditional dishes and recipes, as an ingredient in vegan milks, and as walnut oil. Walnuts are recognized as oilseeds, with fat content ranging from 52 to 70%. In recent years, there has been a surge in walnut oil consumption as a substitute for other vegetable oils, owing to its distinctive nutritional composition. Walnut oil stands out for its richness in polyunsaturated fatty acids (PUFAs), including a relatively high amount of α-linolenic acid, vitamin E, phytosterols, polyphenols, and its unique flavor profile (Orsavova et al., 2015). However, oil extraction yields and the chemical composition of walnut oil can vary depending not only on edaphoclimatic and genetic factors but also on the extraction method employed. Aqueous enzymatic extraction, ultrasonic extraction, Soxhlet extraction, cold-pressing, and supercritical fluid extraction are some of the methods

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utilized for walnut oil extraction, as reviewed by Song et al. (2022). Among those, walnut oil is traditionally extracted by cold pressing, which ensures high-quality oil production through a solvent-free, cost-effective, and environmentally friendly process, preserving bioactive compounds such as phenolics, tocopherols, and phytosterols (Rabadán et al., 2018).

Generally, the walnut oil extraction process begins with hulling to obtain the kernel. Then, the kernels are mechanically crushed or ground into a coarse paste to break down cell walls, which is submitted to cold-press oil extraction. In this process, a by-product, referred to as walnut oilcake (WOC) or press-cake, emerges after pressing the nuts to extract the oil. This fibrous and protein-rich residue has potential applications in the food and feed industries, not only due to its high nutritional value but also for its potential to enhance the nutritional profile and functional properties of formulated products (Bakkalbasi et al., 2015). The obtained raw oil generally contains fine solid particles. To remove these impurities, the oil is commonly subjected to settling, or centrifuged, and then decanted. During this stage, another by-product is generated, the walnut oil dregs (WOD), which correspond to the residues or sediments formed during decantation and centrifugation processes. These dregs are a dense, semi-solid material composed of fine particles and residual oil. Currently, WOD and WOC are discarded or utilized in low-value applications such as animal feed or as a source of material for combustion (Xu et al., 2020). The food industry is actively pursuing innovations to minimize economic losses and adopt a zero-waste approach. By employing diverse extraction techniques, these by-products can be valorized, offering both health benefits and commercial viability when reintegrated into the food value chain.

The literature reports the walnut oilcake as a source of protein, dietary fiber, carbohydrates, and bioactive molecules (Pop et al., 2020). Despite its nutritional potential, the use of oilcake for human consumption remains largely limited (Pop et al., 2020). However, recent studies have successfully incorporated walnut oilcake as a flour substitute in bakery products, yielding multiple benefits. Its inclusion has been shown to reduce the firmness of cakes, enhance the antioxidant potential of wheat bread, lower production costs, and increase the fiber and protein content in gluten-free formulations. For cakes, the addition of WOC up to 15% has been found to improve their softness (Burbano et al., 2022), likely due to components in the oilcake contributing to this texture. In bread formulations, the incorporation of WOC (3–5%) has been shown to significantly enhance antioxidant potential, reaching up to 3.7 times higher than bread made solely with wheat flour. However, the addition of WOC also influenced the bread's chewiness, with firmness increasing when oilcake exceeded 3% of the formulation (Pycia et al., 2019).

Regarding WOD, recent studies have explored their potential uses, particularly focusing on protein isolation (Liu et al., 2022). While WOD overall contains substantial protein content, low-yield oil extraction methods, such as cold pressing, may generate residues with a high content of lipids, mainly bulk triacylglycerols. Therefore, most studies aiming to utilize or investigate WOD proteins frequently use organic solvents to remove WOD fat content (Hong et al., 2024).

The incorporation of edible agri-food by-products, such as WOD and WOC, into food products represents a promising area of study, with economic, sustainable, technological, and health-related benefits reported for various applications. Although walnut oilcake and dregs have been previously explored for isolated applications such as protein recovery or incorporation into bakery products, comprehensive studies addressing their biochemical composition together with the bioaccessibility and intestinal transport of their polyphenols, and prebiotic potential remain scarce. Therefore, this study aims to provide a detailed nutritional characterization of walnut oilcake obtained after the cold pressing process from a Portuguese industry specialized in the production of walnut oil, as well as of the dregs generated during processing, being the first study to provide an integrated evaluation of these by-products. The extracts prepared from these by-products were characterized for their phenolic compounds composition and bioactive properties, including antioxidant, cytotoxic, anti-inflammatory, and antimicrobial activities. Furthermore, to better understand the potential application of these walnut by-products in foods, *in vitro* bioaccessibility and intestinal absorption of phenolic compounds, as well as the prebiotic activity of duodenal residues were estimated after *in vitro* digestion of WOC and WOD. By integrating chemical, functional, and sustainable aspects, this work addresses a significant knowledge gap and highlights the value of these agri-food by-products as functional ingredients, offering novel insights for both food technology and health-related applications.

2. Material and methods

2.1. Chemicals and reagents

All analytical- and LC/MS-grade solvents and reagents, standards of phenolic compounds, soluble sugars, organic acids, tocopherols, and fatty acids (FAME mix, Supelco FAME-MIX, 47885-U) used in the analyses were purchased from Sigma-Aldrich (St. Louis, USA), unless otherwise stated. Enzymes α-amylase (cat# A1031, experimental activity 756 U/mg solid), pepsin (cat# P6887, experimental activity 2014 U/mg solid), and pancreatin (cat# P7545, experimental activity 10.1 U/mg solid), and bile salts (cat# B8631, 100 mmol/g), were also obtained from Sigma-Aldrich (St. Louis, USA), and their experimental activities were measured right before experiments according to Brodkorb et al. (2019) to ensure reproducibility and compliance with the standardized protocol. The commercial standard tocol was obtained from Matreya (Pleasant Gap, USA).

The cell lines used in the cytotoxicity assays were obtained from the Leibniz-Institute DSMZ (German Collection of Microorganisms and Cell Cultures GmbH), except for the primary cell line PLP2 line. Murine macrophage cells (RAW 264.7) were obtained from European Collection of Authenticated Cell Cultures) and Caco-2 cell utilized in the bioavailability assay were from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS)), and nonessential amino acids (NEAA, 1%) acquired from Gibco (Thermo Fisher Scientific - Waltham, MA, USA), as well as L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL), were used for cell culture. Transwell® plates (12 mm diameter inserts, 0.4 μm pore size; Costar, Corning, Madrid, Spain) were utilized for transport experiments.

2.2. Samples

Walnut oil dregs and oilcake were kindly provided by Quinta da Eira (Lamego, Portugal) and transported after walnut oil processing to the research centre under refrigeration temperature. To obtain the concentrated WOD, the sample was centrifuged at 15,000 x*g*, during 10 min (Thermo Scientific Heraeus Multifuge X1R Centrifuge - Osterode am Harz, Germany). The WOC was grounded to powder (~20 mesh sieve, model A327R1 - Moulinex, Barcelona, Spain). None of the samples was degreased using solvents.

2.3. Nutritional characterization

2.3.1. Proximate composition and energetic value

Moisture was evaluated in a moisture analyzer (PBM, Adam equipment, Oxford, USA). Ashes, total fat, crude protein, total carbohydrates and dietary fiber were determined according to AOAC Official methods (AOAC, 2016). Briefly, ashes were determined by gravimetry after incineration in muffle (550 °C, Lenton ECF 12/22, Hope Valley, UK), crude protein estimated after total nitrogen determination by Kjeldahl method (conversion factor 6.25, Kjeldahl distiller model Pro-Nitro-A, JP Selecta - Barcelona, Spain) and total fat by gravimetry after solvent

extraction in a Soxhlet apparatus (Behr Labor Technik - Dusseldorf, Germany). The determination of soluble, insoluble and total dietary fiber was determined by an enzyme-gravimetric method according AOAC Method 991.43 (AOAC, 2016). Total carbohydrate percentage was determined by difference, subtracting the sum of moisture, ashes, crude proteins, and crude fats, from the total weight (100%). Additionally, available carbohydrates were calculated as the difference between total carbohydrates and dietary fiber.

Energy balance was calculated according to following formula (Eq. (1)):

$$Energy\left(\frac{kcal}{100g}\right) = 4\,x\,(g\,proteins + g\,available\,carbohydrates) + 9\,x\,(g\,crude\,fat) + 2\,x\,(total\,dietary\,fibre) \quad (1)$$

being available carbohydrates = total carbohydrates – total dietary fiber, according to regulation (EU) n° 1169/201, (Publications Office of the European Union, 2011).

2.3.2. Soluble sugar composition

Soluble sugars were extracted from 1 g of defatted sampleusing 80% ethanol and subjected to heating at 80 °C for 90 min. After filtration, the solvent was evaporated, and the remaining residue was reconstituted in a final volume of 5 mL. A high-performance liquid chromatograph (HPLC, Knauer, Smartline system 1000, Berlin, Germany) system equipped with a pump (Knauer, Smartline System 1000, Berlin, Germany), degassing unit (Smartline Manager 5000), autosampler (AS-2057 Jasco, Easton, MD, USA), and a refractive index (RI) detector (Knauer Smartline 2300) was used to separate, identify and quantify the free sugars. Separation was carried out using a Eurospher 100–5 NH_2 column (4.6 × 250 mm, 5 µm, Knauer, Berlin, Germany) using chromatographic parameters previously described by Spréa et al. (2024). Commercial standards were used to identify the peaks, with data being analyzed with the Clarity 2.4 software (DataApex, Prague, Czech Republic). Melezitose (25 mg/mL) was used as internal standard for quantification. Results were expressed in g sugar/100 g of sample fresh weight (fw).

2.3.3. Organic acid composition

Organic acids were analyzed according to a methodology previously described by Pascoalino et al. (2025). An amount of 1 g of each walnut by-product was subjected to room temperature maceration for 20 min with 25 mL of 4.5% metaphosphoric acid, stirred at 150 rpm using magnetic agitation while protected from light. The mixture was then centrifuged at 4000×*g* for 5 min, and the resulting extract was filtered through a 22 µm PVDF membrane. The filtrate was analyzed using ultra-fast liquid chromatography equipped with a diode array detector (UFLC-DAD, Shimadzu 20A series, Kyoto, Japan). Compounds were separated on a C_{18} SphereClone reversed-phase column (Phenomenex, 250 × 4.6 mm, 5 µm) maintained at 35 °C. Elution was performed with 3.6 mM sulfuric acid as the mobile phase at a flow rate of 0.8 mL/min. Compounds were processed at 245 nm (ascorbic acid), and 215 nm (oxalic, quinic, malic, ascorbic, citric and fumaric acids) and compounds identified by comparison with authentic standards. The software LabSolutions Multi LC-PDA (Shimadzu Corporation, Kyoto, Japan), was used to data processing, and results were expressed in g organic acid/100 g sample (fw).

2.3.4. Fatty acid composition

Lipids extracted *via* Soxhlet were derivatized to produce fatty acid methyl esters (FAMEs) using a solution of methanol, sulfuric acid, and toluene (2:1:1, *v/v/v*). Fatty acids were analyzed using gas chromatography with flame ionization detection (GC-FID, YOUNG IN Chromass 6500 GC System, equipped with a Zebron-FAME column (30 m × 0.25 mm, 0.20 µm). Chromatographic parameters were those previously described by Spréa et al. (2024). The relative retention times of the FAMEs obtained from walnut by-products were compared to a standard mixture to enable their identification. The results were expressed as the proportion of each fatty acid relative to the total fat content (%, normalized area).

2.3.5. Tocopherol composition

Tocopherols were extracted as previously described by Barros et al. (2010). Briefly, 500 mg of sample added by 400 µL of internal standard (tocol, 50 µg/mL) solution were subjected to extraction with methanol, followed by hexane. The resulting extract was centrifuged (15.000×*g*, 10 min, 10 °C, Thermo Scientific Heraeus Multifuge X1R Centrifuge, Osterode am Harz, Germany) after the addition of NaCl saturated solution, and the supernatant collected and evaporated under a nitrogen stream. The analysis was performed using an HPLC system equipped with a fluorescence detector (HPLC-FD, FP-2020, Jasco, Easton, USA). Tocopherol isoforms (α-, β-, γ-, and δ-) were identified by comparison to commercial standards, and quantification was conducted by internal calibration with tocol. Data were processed using Clarity 2.4 software, and results were expressed in mg tocopherol isoform/100 g sample (fw).

2.4. Phenolic compound composition

Samples were submitted to a dynamic maceration extraction protocol (80% ethanol, room temperature, 1 h) under stirring (stirrer plate, Multimatic 9-N, Selecta, Barcelona, Spain). Extracts were filtrated (Whatman paper filter No. 4) and the residue reextracted under the same conditions. Extracts were then combined, concentrated in a rotatory evaporator (Büchi R-210, Flawil, Switzerland) and freeze-dried (Freezone 4.5, Labconco, Kansas, MO, USA).

The polyphenol composition was determined by ultra-performance liquid chromatography with diode array and an Orbitrap mass spectrometer detection (HPLC-DAD-ESI/MS; Dionex Ultimate™ 3000 and Orbitrap Exploris™ 120, Thermo Fisher Scientific, San Jose, CA, USA), according to chromatographic and detector conditions described in detail by (Rodrigues et al., 2023). Separation was achieved using a Spherisorb S3 ODS-2 C_{18} column (150 × 4.6 mm, 3 µm, Waters, Milford, CT, USA) kept at 35 °C. UV–Visible (UV–Vis) spectra were acquired within the range of 180 to 700 nm, and chromatograms processed at 280, 330 and 370 nm. In MS, compounds were ionized using an electrospray ion source (ESI) in negative ion mode, and the full MS and data-dependant MS^2 (high-energy collisional dissociation, HCD 30%) scans acquired between 130 and 1500 *m/z*). Data acquisition and processing were performed using Xcalibur® software (Thermo Finnigan, Thermo Fisher Scientific, San Jose, CA, USA). Identification was performed by analysing retention times, and UV–Vis, MS and MS/MS spectra in comparison with available standards, spectral libraries and pertinent literature. Quantification was performed by external calibration, with analytical curves of gallic acid (1–89 µg/mL), 4-hydroxybenzoic acid (1–83 µg/mL), caffeic acid (1–95 µg/mL), ellagic acid (1–45 µg/mL), apigenin-7-*O*-glucoside (1–45 µg/mL). Results were expressed in mg/g of freeze-dried extract or sample (dry weight, dw).

2.5. In vitro digestion

The gastrointestinal digestion was simulated *in vitro* based on the standardized INFOGEST 2.0 protocol (Brodkorb et al., 2019), comprising the oral, gastric, and small intestinal phases of digestion. Approximately 2.5 g of each walnut by-product was mixed with simulated salivary fluid to create a paste-like consistency to be used as the starting material for digestion. Enzyme and bile solutions were prepared right before use to ensure correct concentrations. The oral phase was conducted for 2 min at 37 °C on an orbital shaker at 180 rpm. To simulate salivary enzyme activity, 600 µL of α-amylase solution (100 U/mL) was added. In the next step, to simulate gastric digestion, pH was adjusted to 3.0; 2.5 mL of pepsin solution (25 mg/mL) and gastric lipase

(160 mg/mL) was added to the mixture. The samples were incubated under the same conditions described in the oral phase (37 °C, 180 rpm) for 60 min. After gastric digestion, the intestinal phase was initiated by adjusting the pH to 6.0 using 1 M $NaHCO_3$. A solution of pancreatin (2 g/L) and bile salts (12 g/L) was added at a concentration of 250 μL/mL to simulate the pancreatic enzyme activity and bile role in the small intestine. The samples were incubated at 37 °C on an orbital shaker at 180 rpm for 120 min. After each digestion phase, the reaction tubes were incubated in a 90 °C water bath for 5 min to stop the reaction.

A cellulose acetate dialysis tube with a molecular weight cut-off of 3 kDa (Spectra/Pro, Spectrum Lab, Breda, Netherlands) was used. This membrane allowed molecules smaller than 3 kDa to pass through. The dialysis tube containing the sample solution was submerged in a water container and stirred at 1000 rpm at room temperature for 24 h. The solution that diffused through the membrane represented the bioaccessible fraction, while the remaining solution inside the dialysis tube represented the colon-available fraction (colonic residue), which was not accessible for absorption at the duodenal level. The bioaccessible fraction was freeze-dried, subjected to solid-phase extraction (SPE, Strata-X from Phenomenex). The column was conditioned with 1 mL of methanol containing 1% formic acid, followed by 1 mL of an aqueous solution with 1% formic acid, with each step repeated three times. The diluted sample, prepared in a 1:1 (*v/v*) ratio with the aqueous 1% formic acid solution, was then loaded onto the column. The elution was performed with 2 mL of the same aqueous formic acid solution. The collected fraction was subsequently dried under a nitrogen stream and reconstituted in 0.1% formic acid aqueous solution for HPLC analysis according to section 2.4 to estimate the *in vitro* bioaccessibility of walnut by-product compounds. This bioaccessible fraction was calculated as the relative proportion (percentage) of phenolic compounds of the non-digested samples (starting materials, namely WOC and WOD) that was quantified in the aqueous bioaccessible fraction after *in vitro* digestion, according to Eq. (2):

$$Bioaccessibility\ (\%) = \left(\frac{[polyphenols]bioaccessible\ fraction}{[polyphenols]walnut\ byproduct}\right)x100 \qquad (2)$$

The freeze-dried bioaccessible fraction was also used in antioxidant assays, whereas the colonic residue was freeze-dried to be used in the prebiotic activity assay.

2.6. Bioactive properties of polyphenol-rich extracts and bioaccessible fractions from walnut by-products

2.6.1. Antioxidant capacity

Two *in vitro* protocols were performed to assess the antioxidant potential of the extracts, namely, the formation of thiobarbituric acid reactive substances (TBARS), and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, according to the protocols described previously by Spréa et al. (2024). Freeze-dried extracts obtained from maceration (see Section 2.4) and freeze-dried bioaccessible fractions (see Section 2.5) from simulated digestion were re-suspended in water at known concentrations and serially diluted to prepare solutions for the assays. Extraction solvent was employed in the blank assay, and Trolox was used as the positive control for both the DPPH and TBARS assays. Additionally, commercial food antioxidants, including calcium ascorbate (E-302) and sodium metabisulfite (E-223), were also employed as positive controls. Results were expressed in IC_{50} (mg/g of extract), corresponding to the concentration required to inhibit 50% of the oxidative processes.

2.6.2. Cytotoxic potential

Three human tumor cell lines were tested, namely the human gastric epithelial cell line (AGS), human colorectal adenocarcinoma (Caco-2), breast carcinoma (MCF-7), and a non-tumoral cell line PLP2 (porcine liver primary culture). All cells were obtained from the Leibniz-Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH, except for the PLP2 line (established and maintained in the laboratory) and used to determine the cytotoxicity potential by Sulforhodamine B assay as previously described by Mandim et al. (2019). Results were expressed in GI_{50}, μg/mL (ability to inhibit 50% of the cell growth). Ellipticine was used as a positive control.

2.6.3. Anti-inflammatory potential

The assessment of the anti-inflammatory effects of freeze-dried WOC and WOD extracts was performed following the methodology described by Mandim et al. (2019). The anti-inflammatory activity was determined *in vitro* using murine macrophage cells (RAW 264.7, European Collection of Authenticated Cell Cultures) by measuring the inhibition of nitric oxide (NO) production. Cells were cultured in DMEM supplemented with 10% FBS and glutamine at 37 °C in a humidified environment with 5% CO_2. For the assay, cells were seeded in 96-well plates at a density of 150,000 cells per well and allowed to adhere for 18 h. Following this, cells were treated with the extract at various concentrations for 1 h. Basal NO levels in the absence of lipopolysaccharide (LPS) were also evaluated using the Griess Reagent System (Promega). Cell culture supernatants (100 μL) were mixed with sulfanilamide and NED solutions, incubated at room temperature for 5–10 min, and the nitrite content was quantified spectrophotometrically at 515 nm. Dexamethasone was used as a positive control. Results were expressed in IC_{50} (half-maximal inhibitory concentration expressed in μg freeze-dried extract/mL). No LPS was used in the negative control to assess baseline conditions without immune activation.

2.6.4. Antimicrobial potential

The antimicrobial activity was evaluated using an adapted broth microdilution assay, following the guidelines of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2018) and incorporating the colorimetric test with *p*-iodonitrotetrazolium chloride as described by Kuete et al. (2011). The assay was performed against eight bacterial strains, including five Gram-negative bacteria (*Enterobacter cloacae* ATCC 49741, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enterica* ATCC 13076, and *Yersinia enterocolitica* ATCC 8610) and three Gram-positive bacteria (*Bacillus cereus* ATCC 11778, *Listeria monocytogenes* ATCC 19111, and *Staphylococcus aureus* ATCC 11632). Streptomycin, methicillin, and ampicillin were included as positive controls.

The antifungal activity was assessed using a microdilution method as described by de la Fuente et al. (2022), targeting two micromycetes, *Aspergillus brasiliensis* (ATCC 16404) and *Aspergillus fumigatus* (ATCC 1022). Ketoconazole was used as the positive control. Results were expressed in mg/mL corresponding to Minimum Inhibitory Concentration (MIC), Minimum Fungicidal Concentration (MFC) and Minimum Bactericidal Concentration (MBC).

2.7. Intestinal bioavailability of polyphenols using the Caco-2 cell model

The intestinal absorption of polyphenols was assessed using the human intestinal Caco-2 cell line to evaluate polyphenol transport across the epithelial barrier and/or accumulation within the cells, as previously described by D'Antuono et al. (2015) with some modification.

2.7.1. Determination of non-toxic concentration

The non-toxic concentration to be used on the bioavailability assay using Caco-2 cells was determined using the MTT assay, following the methodology described by Arranz et al. (2015), with modifications. Caco-2 cells were plated in 96-well plates at a density of 1.5×10^4 cells per well and incubated at 37 °C for 72 h to allow for adhesion and proliferation. Dulbecco's Modified Eagle Medium (DMEM) and Hank's Balanced Salt Solution (HBSS) were used as controls to assess potential cytotoxic effects. After the incubation period, the cells were washed with PBS, and 0.5 mg/mL of MTT solution was added to each well. The plates

were incubated at 37 °C for 4 h, allowing for the formation of formazan crystals. Following incubation, the supernatants were carefully removed, and the insoluble formazan crystals were dissolved in DMSO. Absorbance was measured at 570 nm, and the results were expressed as a percentage relative to untreated control cells. Extract concentrations that maintained cell viability above 80% were considered non-toxic under these conditions.

2.7.2. Uptake and basolateral transport experiments on Caco-2 cells

For the uptake experiments, Caco-2 cells (ATCC, Manassas, VA, USA) were cultured in DMEM supplemented with fetal bovine serum (10%), penicillin (100 U/mL), streptomycin (100 μg/mL), nonessential amino acids (1%), and L-glutamine (2 mM). The cells were maintained at 37 °C in a 5% CO_2 humidified incubator. Transport studies were performed using Transwell® plates (12 mm diameter inserts, 0.4 μm pore size, Costar, Corning, Madrid, Spain), with cells seeded at 2.2×10^6 cells/insert and cultured for 21 days. The culture medium was replaced every two days, and transepithelial electrical resistance (TEER) was monitored. Only inserts with TEER values >1000 Ω·cm^2 were used.

For the transport experiments, the apical and basolateral compartments were preconditioned with DMEM (without FBS) for 30 min. 500 μL of digested extracts were added to the apical compartment and incubated for 4 h at 37 °C. TEER values were measured before and after incubation to confirm monolayer integrity.

Samples from the apical and basolateral compartments were freeze-dried and stored at −20 °C for later analysis. Phenolic compound analysis followed the methodology of Villalva et al. (2022), with modifications. Lyophilized apical and basolateral samples were extracted with 100 μL of 80:20 ethanol:water solution (*v/v*), centrifuged for 5 min at 10,000 rpm, and filtered through 0.22 μm filters before HPLC analysis. For the cell monolayer harvesting, cells were rinsed with cold PBS, treated with 500 μL of 80:20 hydroalcoholic solution, and incubated at 4 °C for 30 min. The cells were then scraped, sonicated for 5 min, and centrifuged at 4500 rpm for 15 min. This process was repeated three times, and the combined supernatants were evaporated under nitrogen gas. The residue was re-dissolved in 100 μL of 80:20 ethanol:water solution (*v/v*), filtered, and subjected to HPLC analysis as described in Section 2.4.

2.8. Prebiotic activity

Six commercially available probiotic strains were selected to evaluate the prebiotic activity. The strains included *Lactobacillus rhamnosus* R11, *Bifidobacterium animalis* B0, *Bifidobacterium longum* BG3, *Lactobacillus casei* L01, *Lactobacillus acidophilus* LA-5, and *Bifidobacterium animalis* ssp. *lactis* Bb12. Undigested walnut by-products and the colonic residue obtained upon *in vitro* digestion were diluted in De Man, Rogosa, and Sharpe (MRS) broth at a 2:100 ratio (*w/v*). Fructooligosaccharides (FOS) and glucose were used as positive controls. Incubation and growth conditions followed the methodology described by Gómez-García et al. (2022) with slight modifications. Samples were filtered into Eppendorf tubes using a sterile syringe filter (22 μm). A volume of 15 μL from each probiotic inoculum was added to 735 μL of each sample and gently mixed. Subsequently, 200 μL of the mixture was transferred to a microplate, and, for samples inoculated with *Bifidobacterium* species, 50 μL of liquid paraffin was added to the top of each well to create an anaerobic environment. The microplate was sealed with parafilm and incubated at 37 °C for 48 h under agitation. Absorbance measurements were recorded at 620 nm at hourly intervals throughout the incubation period to monitor bacterial growth.

2.9. Statistical analysis

All the analyses were performed at least in triplicate, and the results were expressed as mean ± standard deviation (SD). Statistical analyses were performed using SPSS Statistics (version 23). A Student's *t*-test was applied at a 95% confidence level ($p < 0.05$) to compare the means of the results between WOC and WOD.

3. Results and discussion

3.1. Chemical characterization of walnut by-products

As expected, the proximate composition of WOD and WOC was characterized by a low moisture content (1.8 ± 0.2 and 8.5 ± 0.3 g/100 g fw, respectively, Table 1). Lipids comprised the main macronutrient class in WOD (45.6 ± 0.9 g/100 g fw). The high total fat content of WOD was expected since cold-press extraction was employed to obtain the oil from walnut kernels, which has the advantage of not using solvents but generally results in lower extraction yields. Before carrying out the proximate analysis, a centrifugation step was performed to separate the bulk oil in the sample, resulting in 30% of oil recovery after the centrifugation process. In case the industry decides to include a centrifugation step of the dregs, this oil can be further recovered as cold-pressed walnut oil. In addition to the high content of lipids, the dregs after centrifugation were also found to have considerable amounts of available carbohydrates and proteins (26 ± 1 and 10.9 ± 0.4 g/100 g

Table 1
Nutritional composition, energetic value, free sugars, organic acids, fatty acids and tocopherols of walnut by-products.

	WOC	WOD
Nutritional Composition		
Moisture	8.5 ± 0.3 ^{b}	1.8 ± 0.2^a
Ashes	0.1 ± 0.01 ^{b}	3.3 ± 0.2 ^{a}
Crude protein	38.1 ± 0.1 ^{b}	10.9 ± 0.2 ^{a}
Total fat	7.8 ± 0.4 ^{b}	45.6 ± 0.9 ^{a}
Solube dietary fiber	2.6 ± 0.1 ^{b}	1.6 ± 0.1 ^{a}
Insoluble dietary fiber	30 ± 1 ^{b}	11.3 ± 0.1 ^{a}
Available carbohydrates	12.9 ± 0.4 ^{b}	25.5 ± 1 ^{a}
Energy (kcal/100 g)	341 ± 3 ^{b}	578.7 ± 4.4 ^{a}
	Free Sugars (g/100 g fw)	
Sucrose	9 ± 1 ^{b}	13.1 ± 0.6 ^{a}
Total free sugars	9 ± 1 ^{b}	13.1 ± 0.6 ^{a}
	Organic Acids (g/100 g fw)	
Oxalic acid	0.3 ± 0.01 ^{a}	0.2 ± 0.01 ^{a}
Quinic acid	1.8 ± 0.1^b	1 ± 0.1 ^{a}
Malic acid	0.8 ± 0.1 ^{b}	0.3 ± 0.01 ^{a}
Citric acid	2.3 ± 0.01 ^{b}	0.3 ± 0.01 ^{a}
Total organic acids	4.4 ± 0.1 ^{b}	1.8 ± 0.1 ^{a}
Fatty acids (relative %)		
16:0 (Palmitic acid)	8.6 ± 0.1 ^{b}	7.7 ± 0.1 ^{a}
18:0 (Stearic acid)	3.8 ± 0.1 ^{b}	3.3 ± 0.1 ^{a}
18:1n-9c (Oleic acid)	14.9 ± 0.3 ^{b}	16.8 ± 0.1 ^{a}
18:2n-6t (*trans*-Linoleic acid)	nd	0.21 ± 0.01
18:2n-6c (Linoleic acid)	58 ± 1^a	56.8 ± 1 ^{a}
18:3n-3 (alpha-Linolenic acid)	14.7 ± 1^a	14.7 ± 0.1 ^{a}
20:0 (Arachidic acid)	nd	0.2 ± 0.01
20:1 (Gondoic acid)	nd	0.3 ± 0.01
SFA	12.4 ± 0.2	11.3 ± 0.2
MUFA	14.9 ± 0.3	17.1 ± 0.1
PUFA	72.7 ± 0.2	71.1 ± 1.1
	Tocopherols (mg/100 g fw)	
α-Tocopherol	0.8 ± 0.1 ^{a}	0.6 ± 0.1^a
γ-Tocopherol	22.4 ± 0.6 ^{b}	20.4 ± 0.7 ^{a}
δ-Tocopherol	1.85 ± 0.01 ^{b}	1.45 ± 0.01 ^{a}
Total Tocopherol	25.05 ± 0.71	22.45 ± 0.81

Data are presented as mean ± SD ($n = 3$). Fatty acid results are expressed in percentage (normalized chromatographic area). WOD: walnut oil dregs. WOC: walnut oilcake. nd: not detected; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. Different letters in the same row indicate significant differences (*t*-test, $p < 0.05$) between samples.

fw, respectively). Previous studies on the use of WOD focused on protein extraction, often requiring a previous step using organic and toxic solvents to degrease the dregs, since their high fat content interferes with protein recovery (Hong et al., 2024). Overall, a high content of lipids was also reported in the previous studies. Regarding carbohydrates, which represent the second most abundant component, insoluble dietary fiber (IDF) and soluble dietary fiber (SDF) accounted for 11.3 ± 0.1% and 1.6 ± 0.1%, respectively, in WOD. Both IDF and SDF have been reported to play relevant roles in human nutrition, promoting intestinal health and supporting gut microbiota (Tang et al., 2024). Similarly, WOC showed to be rich in fiber, exhibiting significantly higher fiber content compared to WOD, with IDF and SDF contributing about 30 ± 0.1% and 2.6 ± 0.1%, respectively, reinforcing its potential as a valuable dietary fiber source. In contrast to WOD, WOC stands out for its high protein content (38.1 ± 0.1 g/100 g fw). Moreover, it also presented a high carbohydrate content (45.5%), although most of it was composed of fiber as mentioned (total of 32.6 ± 1.0 g/100 g fw, primarily insoluble fiber with 30 ± 1 g/100 g fw). Regarding the available carbohydrates, the content of free sugars was 9 ± 1 g/100 g fw with only sucrose being detected (Table 1). Sucrose was also the only free sugar detected in WOD, although in slightly higher amounts (13.1 ± 0.6 g/100 g fw).

Among the few studies reporting the nutritional composition of walnut oilcake found in the literature, Burbano and Correa (2021) reported the total fat to be the main macronutrient (55.7%), followed by protein (24.6%). The high-fat content of the oilcake suggests a low oil extraction yield, which may be related to the sample preparation (*i.e.*, inclusion or not of a kernel paste preparation and corresponding particle dimension) and the extraction method used (hydraulic cold extraction). Similarly, Santos et al. (2018) reported crude fat content of walnut oilcakes varying between 18% and 28%, higher than that found in the present study (7.8%). These differences may be attributed to the fact that Santos et al. (2018) used a small-scale process, with only 500 g of sample being subjected to pressing, whereas the present study analyzed a by-product obtained from industrial processing. Overall, the discrepancies in the values of fat content can be explained not only by the scale of processing or sample preparation but also by additional agronomic and technological factors, such as walnut cultivar, kernel maturity at harvest, and moisture content, all of which influence oil release. Post-harvest storage conditions, including time and temperature, may further alter oil stability and extraction efficiency. Moreover, industrial cold-pressing generally achieves lower residual fat compared to small-scale or hydraulic extractions, which are less efficient. These combined aspects likely account for the striking variability in fat content reported across studies. Similarly to the current work, Pop et al. (2020) evaluated walnut oilcake as an ingredient in pastry formulations and reported carbohydrates as the main macronutrient. However, the authors did not quantify dietary fiber separately, incorporating it within the total carbohydrate content. However, they quantified the fiber content in macarons produced with increasing amounts of walnut oilcake observing an increase in fiber with higher levels of oilcake inclusion. Beyond its nutritional value, walnut oilcake has also attracted interest for protein recovery. For instance, Grosso et al. (2020) reported that proteins from the oilcake can be used as a component for the development of an edible antioxidant coating to improve walnut shelf life.

Overall, the findings obtained in the current study align with the literature. Based on the differences in the nutritional composition of the two by-products, the WOD can be used as a potential source of fat, carbohydrates and fibers, while WOC can be exploited as a source of proteins and fibers.

Organic acids are present in different plants, playing important roles in plant metabolism and in microbial interaction. Moreover, organic acids confer specific sensory characteristics (generally acidifying), being compounds of interest to the food industry. A total of 1.8 ± 0.1 g/100 g fw of organic acids were present in WOD, while 4.4 ± 0.1 g/100 g fw were identified in WOC. Quinic acid was the most predominant in WOD, followed by malic, oxalic, and citric acid, while citric acid stood out in the oilcake sample, reaching 2.3 g/100 g fw, followed by quinic, malic, and oxalic acid.

Regarding fatty acid composition (Table 1), both WOD and WOC samples presented a similar profile with polyunsaturated fatty acids as the predominant compounds. Linoleic acid was the most abundant fatty acid, representing 58.0 ± 1.0% in WOC and 56.8 ± 0.6% in WOD. Oleic acid was the main monounsaturated fatty acid in both samples, with values of 14.9 ± 0.3% and 16.8 ± 0.2% for WOC and WOD, respectively. Both samples presented relevant contents of α-linolenic acid, which has been positively associated with health benefits for its anti-inflammatory effect and cardiovascular protection. Overall, the obtained results align with those previously reported in walnut by-products (Bakkalbasi et al., 2015; Pop et al., 2020), as well as in raw walnuts (Pascoalino et al., 2025), suggesting that walnut by-products can be a source of fatty acids and potential new ingredients to food industry.

Vitamin E isoforms (with α-tocopherol being the most active) have a crucial role as primary and secondary antioxidant, specifically protecting PUFAs from oxidation, breaking the chain reaction induced by peroxyl radicals, and regenerating other antioxidants. Table 1 presents the tocopherol composition of walnut oil processing by-products. Three isoforms of tocopherols were identified, with γ-tocopherol (22.4 ± 0.6 mg/100 g fw) being the main isoform in WOD, and WOC (20. ± 0.7 mg/100 g fw), in agreement with previously reported results (Bakkalbasi, 2019; Bakkalbasi et al., 2015; Santos et al., 2018). The profile obtained for both by-products is also similar to that of raw walnuts reported in previous works (Pascoalino et al., 2025).

3.2. Phenolic compounds composition of walnut by-products

A total of 41 compounds were tentatively identified in the hydroethanolic extracts obtained from both by-products of walnut oil processing (Table 2, Fig. 1). The polyphenol profile was similar between WOC and WOD, characterized primarily by hydrolysable tannins, dicarboxylic acid derivatives characteristic of walnuts, besides phenolic acids and their glycosides. On the other hand, in quantitative terms, the total phenolic content (TPC, sum of compounds quantified by HPLC-DAD) was almost three times higher ($p < 0.05$) in WOC (32.03 ± 0.20 mg/g freeze-dried extract) than in WOD (12.04 ± 0.02 mg/g freeze-dried extract), as can be noticed by the different chromatographic signal of both extracts analyzed under the same conditions in Fig. 1A and B. As an earlier by-product of the walnut oil production, walnut oilcake likely retained a greater diversity and concentration of phenolic compounds than the material eventually taken with the walnut oil after kernel pressing, which is later sedimented. The amount of extractable phenolic compounds found corroborates that walnut by-products, particularly the oilcake, retain bioactive compounds and can be better explored.

Glansreginin A (peak 17), was the major compound found in both samples, accounting for 21% (6.81 ± 0.06 mg/g extract dw) and 28% (3.34 ± 0.00 mg/g extract dw) of TPC in WOC and WOD, respectively. This compound has been reported as the major compound found in walnut kernels (Slatnar et al., 2015) and peeled walnut kernels (Medic et al., 2021), and is regarded as a quality indicator for this product, besides being associated with anti-inflammatory and antioxidant properties (Gao et al., 2019; Haramiishi et al., 2020). Glansreginin A presented an intense molecular ion $[M–H]^-$ at *m/z* 592, forming diagnostic fragments ions at *m/z* 403, 343, 283, 241, 223, 197, 188, 181, 179, and 144, as described by Ren et al. (2022),which characterized the defatted walnut powder extract also using Orbitrap as mass analyzer. With the same UV–Vis and MS characteristics of peak 17, peak 19 was identified as glansreginin A isomer. Moreover, peaks 22 ($[M–H]^-$ at *m/z* 572) and 24 ($[M–H]^-$ at *m/z* 574) eluted later and presented a mass difference of 20u (H_2O—2H) and 18u (H_2O) in their ions lower than those of glansreginin A, as also noticed in walnut extracts (Ren et al., 2022), and were

Table 2
Tentative identification and quantification of phenolic compounds of walnut oil processing by-products.

Peak[a]	RT (min)[b]	λ_{max} (nm)[c]	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative Identification	Quantification (mg/g extract dw)[d]	
						WOC	WOD
1a	4.01	268	169	125	Gallic acid	5.34 ± 0.30[a]	1.51 ± 0.04[b]
1b			368	144	1,2,3,4-Tetrahydro-2-oxo-4-quinolinecarboxylic acid 4-*O*-D-glucoside		
1c			951	783, 481, 301	HHDP-valoneoyl-glucose		
2a	4.22	276	783	481, 301, 275, 257	Bis-HHDP-glucose	0.92 ± 0.04[a]	0.20 ± 0.01[b]
2b			443	281, 237, 189, 161	Dihydrophaseic acid hexoside		
3a	4.52		785	633, 483, 301	Digalloyl-HHDP-glucose	1.12 ± 0.08[a]	0.16 ± 0.01[b]
3b			401(355 + FA)	193, 179, 149, 135	Ferulic acid-*O*-hexoside		
3c			680	269, 209, 167, 144	ni		
4a	5.19	265	291	247, 219, 191	Brevifolin carboxylate	0.42 ± 0.01[a]	0.20 ± 0.01[b]
4b			951	783, 481, 301	HHDP-valoneoyl-glucose		
4c			419	177, 133	Dihydroxy-coumarin derivative		
5a	6.32	256, 278sh, 344	259	241, 197, 159	Dicarboxylic acid derivative	0.20 ± 0.01[a]	0.12 ± 0.01[b]
5b			463	301, 257, 244, 229	Ellagic acid-*O*-hexoside		
6a	6.6	274	137	108	*p*-Hydroxybenzoic acid	5.85 ± 0.06[a]	1.75 ± 0.00[b]
6b			979(933 + FA)	451, 301, 275	Glansrin C		
6c			785	633, 483, 301	Digalloyl-HHDP-glucose		
6d			401	269, 161	Benzyl-pentosylhexoside		
6e			727	403, 325, 265, 223, 179	Glansreginin B hexoside		
7a	7.23	260(sh), 300sh	635	465, 313, 169, 125	Trigalloyl-glucose	0.06 ± 0.01[a]	0.04 ± 0.01[a]
7b			431(385 + FA)	205, 153	Roseoside		
8a	7.69	267	423	243, 199, 169	Glansreginic acid 8-*O*-β-D-glucoside + H_2O + 2H	0.38 ± 0.02	nd
8b			933	451, 301	Glansrin C isomer		
8c			1103	301, 275	Glansrin A		
9a	8.21	268	281	237, 189, 171, 123	Dihydrophaseic acid	0.94 ± 0.03[a]	0.09 ± 0.01[b]
9b			935	633, 463, 301	Galloyl-bis-HHDP-glucose		
10	10.19	271	565/1131	403, 343, 293, 283, 241, 197, 181	Glansreginin B	1.45 ± 0.07[a]	1.05 ± 0.01[b]
11	10.60	254, 360	433/867	301, 229, 244, 257	Ellagic acid-*O*-pentoside	2.16 ± 0.14[a]	0.48 ± 0.01[b]
12a	10.91	256, 280(sh), 360	633	403, 343, 305, 263, 237, 144	Dicarboxylic acid derivative	0.73 ± 0.03[a]	0.38 ± 0.01[b]
12b			922	623, 494, 409, 289, 245, 229, 173	Procyanidin		
13	13.23	254, 367	301	257, 245, 229	Ellagic acid	1.35 ± 0.13[a]	0.69 ± 0.03[b]
14	13.76	256, 363	403/807	223, 179	Glansreginic acid-*O*-hexoside	0.97 ± 0.04[a]	0.53 ± 0.02[b]
15	14.55	258, 366	754	403, 325, 265, 223, 179, 144	Glansreginin A hexoside	0.50 ± 0.01[a]	0.18 ± 0.01[b]

(*continued on next page*)

Table 2 (*continued*)

Peak[a]	RT (min)[b]	λ_{max} (nm)[c]	[M-H]$^-$ (*m/z*)	MS^2 (*m/z*)	Tentative Identification	Quantification (mg/g extract dw)[d]	
						WOC	WOD
16a	15.00		469	169	Valoneic acid dilactone	0.87 ± 0.01[a]	0.07 ± 0.01[b]
16b			939	769, 617, 465, 447, 431, 169, 125	Penta-*O*-galloyl glucose		
17	17.60	268	592/1185	403, 343, 283, 241, 223, 197, 179, 144	Glansreginin A	6.81 ± 0.06[a]	3.34 ± 0.01[b]
18	17.96	268	241	197, 181	Glansreginic acid	0.12 ± 0.01[a]	0.14 ± 0.01[a]
19	19.42	269	592	403, 343, 283, 241, 197, 144	Glansreginin A isomer	0.25 ± 0.01[a]	0.17 ± 0.01[a]
20	20.84	265, 368	943	592, 403, 343, 283, 241, 197, 144	Glansreginin C	0.18 ± 0.01[a]	0.09 ± 0.01[b]
21a	21.34	260, 355, 368	923	323, 263, 177, 144	ni	0.20 ± 0.01[a]	0.15 ± 0.01[b]
21b			555/577	169, 125	ni		
22	23.06	252, 353, 368	572	263, 221, 177, 144, 133	Glansreginin A–H_2O–2H	0.36 ± 0.01	0.27 ± 0.01[b]
23	25.55	268	978	627, 403, 343, 283, 241, 197, 144	Dicarboxylic acid derivative	0.20 ± 0.01[a]	0.09 ± 0.01[b]
24	27.31	267	574	429, 325, 265, 221, 193, 179, 144	Glansreginin A-H_2O	0.31 ± 0.01[a]	0.18 ± 0.01[b]
25	27.74	267	627	403, 343, 283, 241, 197	Dicarboxylic acid derivative	0.33 ± 0.01[a]	0.15 ± 0.01[b]
					Total phenolic compounds	32.03 ± 0.20 [a]	12.04 ± 0.02 [b]

ni: not identified; nd: not detected; FA: formic acid adduct. WOD: walnut oil dregs. WOC: walnut oilcake. Different letters in the same row indicate significant differences ($p < 0.05$) between samples.

[a] Peaks are numbered according to the chromatogram shown in Fig. 2. More than one compound per peak, as indicated by letters after the peak number, refers to compound coelution within the same chromatographic peak.

[b] Retention time on C_{18} column.

[c] gradient of 0.1% formic acid in water and acetonitrile.

[d] Compounds are expressed as mean ± SD (mg/g freeze-dried extract).

tentatively identified as glansreginin A-H_2O-2H and glansreginin A-H_2O, respectively. Peak 15 ([M–H]$^-$ at *m/z* 754) presented a MS^2 fragment ion [M–H-162]$^-$ at *m/z* 592, corresponding to the glansreginin A molecule and to the loss of a hexose moiety, besides similar fragments to those found in that compound, being then tentatively identified as glansreginin A hexoside. In addition, glansreginin B (peak 10, [M–H]$^-$ at *m/z* 565) and its hexoside (peak 6e, [M–H]$^-$ at *m/z* 727), glansreginin C (peak 20, [M–H]$^-$ at *m/z* 943), glansreginic acid (peak 18, [M–H]$^-$ at *m/z* 241) and its glycosides (peaks 8a, and 14, [M–H]$^-$ at *m/z* 423 and 403), and 1,2,3,4-tetrahydro-2-oxo-4-quinolinecarboxylic acid 4-*O*-D-glucoside (peak 1b, [M–H]$^-$ at *m/z* 368), besides four dicarboxylic acid derivatives (peaks 5a, 12a, 23, and 25), represented the class of dicarboxylic acids, commonly found in walnuts, in samples analyzed. The identification parameters of these compounds presented in Table 2 were in line with those MS and MS/MS characteristics previously described in the pertinent literature for their identification (Medic et al., 2021; Regueiro et al., 2014; Ren et al., 2022; Slatnar et al., 2015).

As for glansreginin A, glansreginin B was also present in substantial amounts in both samples, being the fourth (1.05 ± 0.01 mg/g) and the fifth (1.45 ± 0.01 mg/g extract dw) major compound found in WOD and WOC, respectively. Precisely, one of the differences between the phenolic profile of WOD and WOC was the relative proportion of glansreginin B (peak 10), which accounted for roughly 10% of the TPC in WOD and 5% in WOC, as shown in the overlapping of normalized chromatograms in Fig. S1.

Besides dicarboxylic acids, phenolic acids were also present in high amounts in both samples. The hydroxybenzoic acid derivatives gallic acid (peak 1a, [M–H]$^-$ at *m/z* 169), *p*-hydroxybenzoic acid (peak 6a, [M–H]$^-$ at *m/z* 137), and ellagic acid (peak 13, [M–H]$^-$ at *m/z* 301) were identified by comparison of their UV–Vis and MS spectra and retention time with those of their commercial standards. These were among the major compounds in both samples. Ellagic acid-*O*-hexoside (peak 5c, [M–H]$^-$ at *m/z* 463) and ellagic acid-*O*-pentoside (peak 11, [M–H]$^-$ at *m/z* 433) presented characteristic fragmentation pattern yielding MS^2 ions at *m/z* 301, 257, and 229 after neutral losses of 162u (hexose moiety) and 132u (pentose moiety) from their respective molecular ions (Pycia et al., 2019). The latter was the fourth major compound in WOC (2.16 ± 0.14 mg/g, against 0.48 ± 0.00 mg/g extract dw

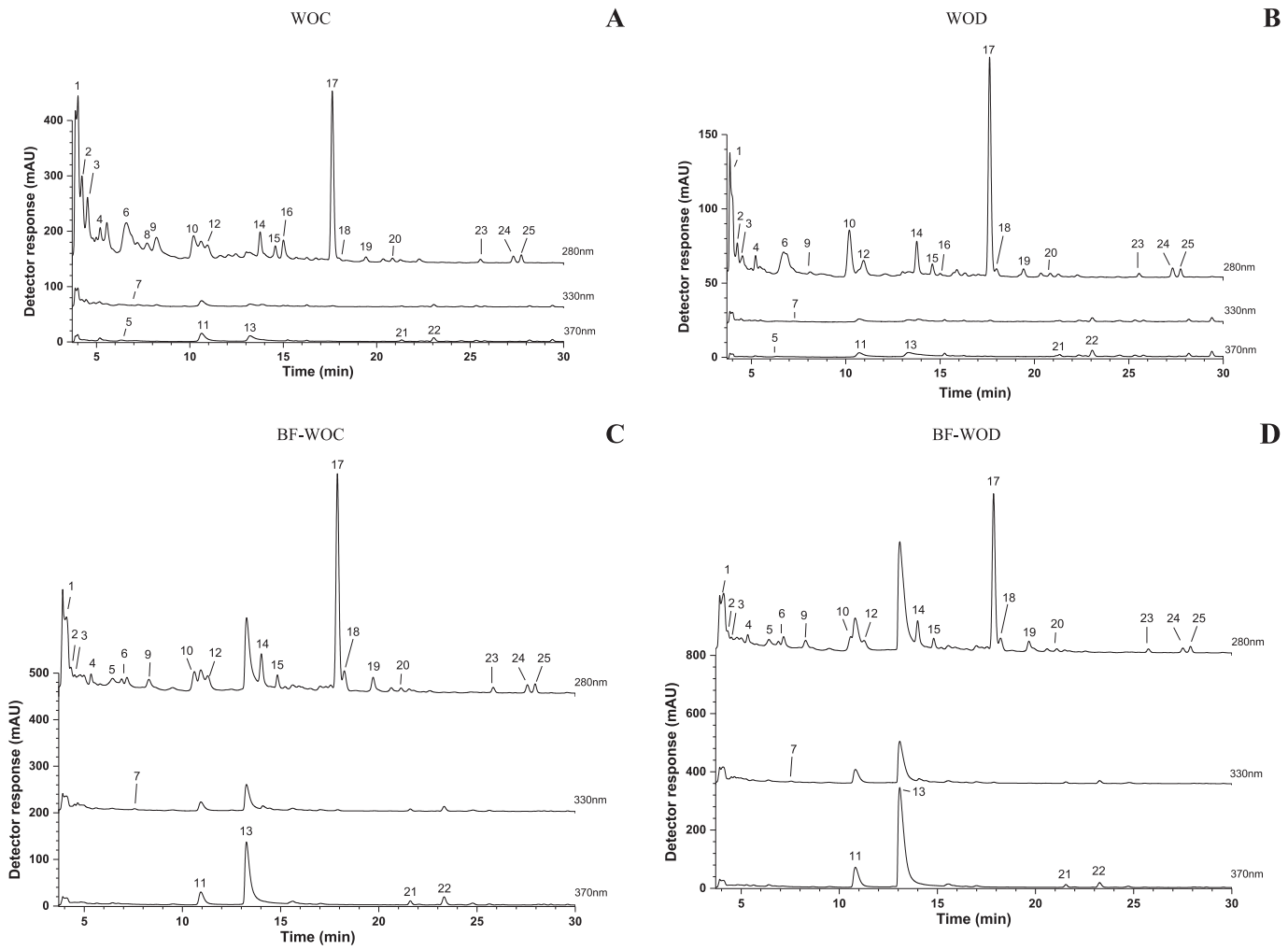

Fig. 1. Chromatograms (HPLC-DAD) of hydroethanolic extracts of polyphenols obtained from A. walnut oilcake (WOC), and B. walnut oil dregs (WOD), and of bioaccessible fractions obtained after *in vitro* digestion of C. walnut oilcake (BF-WOC), and D. walnut oil dregs (BF-WOD), processed at 280, 330 and 370 nm. Peaks are numbered according to Table 1.

found in WOD). Another phenolic acid glycoside, ferulic acid-*O*-hexoside (peak 3b) was detected as its formic acid adduct $[M\text{-}H + HCOOH]^-$ at *m/z* 401, generating the fragment ions at *m/z* 355 and 193 corresponding to ferulic acid attached to a hexose moiety and the aglycone.

Several hydrolysable tannins, primarily ellagitanins (peaks 1c, 2a, 4b, 6b, 6c, 8b, 8c and 16a), were identified in both samples. Ellagitannins are complex or even polimeric molecules that can be hydrolysed under specific conditions to release hexahydroxydiphenoyl (HHDP) acid, which readily lactonizes to ellagic acid. Because of that, neutral losses of one or more HHDP groups (−302 u) or the formation of the diagnostic ion at *m/z* 301 represent one of the characteristic fragmentation pattern of these compounds, along with eventual losses of gallic acid or galloyl moieties that can be esterified to the polyol core of these tannins, and/or the neutral loss of the sugar residue itself, mostly glucose (Moilanen et al., 2013). Two isomers found in peaks 3a and 6c ($[M–H]^-$ at *m/z* 785) presented the ion at *m/z* 301 as the MS^2 base peak, corresponding to HHDP group, besides the ions at *m/z* 633 and 483 indicating the losses galloyl moieties (−152 u) and glucose. Based on these characteristics, also observed by Jia et al. (2018), these compounds were tentatively identified as digalloyl-HHDP-glucose isomers. Bis-HHDP-glucose (peak 2a), displayed an $[M–H]^-$ at *m/z* 783, with fragment ions at *m/z* 481, indicating the loss of one HHDP group, and at *m/z* 301, representing the loss of one HHDP and glucose group (482u). Similar fragmentation was also noticed in peak 9b ($[M–H]^-$ at *m/z* 935), whereas the neutral loss of 634u indicated the presence of an additional galloyl unit in relation to peak 2a, and therefore this compound was assigned as galloyl-bis-HHDP-glucose. Moreover, peaks 1c and 4b, ($[M–H]^-$ at *m/z* 951), presented fragment ions at *m/z* 783 and *m/z* 481, besides the MS^2 base peak at *m/z* 301, and were tentatively identified as HHDP-valoneoyl-glucoside (Moilanen et al., 2013).

Interestingly, hydrolysable tannins characteristically found of walnuts (Ito et al., 2007), glansrin A (peak 8c, $[M–H]^-$ at *m/z* 1103) and glansrin C (peaks 6b and 8b, $[M–H]^-$ at *m/z* 933, or its formic acid adduct at *m/z* 979) were detected only in the oilcake extract (Table 1, Fig. S1). While glansreginins, particularly glansreginin B, and glansreginic acid and its glucoside contributed proportionally more to the WOD phenolic profile, glanrins with higher molecular weights were proportionally more important to WOC profile. These compounds are all prominent markers of the phenolic profile of walnut by-products, and hold potential for their differentiation and singular properties in food or health applications.

Finally, the chemical profile of walnut by-products extracts comprised galloyl glucoses (peaks 7a and 16b), valoneic acid dilactone (peak 16a), brevifolin carboxylate (peak 4a), dihydrophaesic acid in both aglycone and hexoside forms (peaks 2b and 9a), and roseoside (peak 7b), all of which have already been identified in walnuts and their products, besides benzoyl-pentosylhexoside (peak 6d) (Medic et al., 2021; Regueiro et al., 2014; Ren et al., 2022; Slatnar et al., 2015).

3.3. Bioactivities of walnut by-product extracts

3.3.1. Antioxidant activity

Phenolic acids, predominantly present in WOC and WOD samples, are widely recognized for their strong antioxidant potential (Ungar et al., 2003). The results of *in vitro* antioxidant assays performed with hydroethanolic extracts of walnut by-products are presented in Table 3. In the lipid peroxidation inhibition assay (TBARS), WOC exhibited a significantly lower IC_{50} value (9.2 μg/mL) compared to the oilcake (33 μg/mL), indicating a higher inhibition capacity. Both by-products demonstrated greater antioxidant efficacy than commercial food-grade antioxidants, such as E-223 and E-302. Furthermore, WOC showed a greater antioxidant capacity than Trolox (13.6 ± 0.1), a water-soluble analogue of vitamin E. Similarly, in the DPPH assay, WOC showed a markedly lower IC_{50} value (130 μg/mL) compared to WOD (510 μg/mL), reflecting its superior free radical scavenging activity which may be associated with the higher proportion of specific compounds as aforementioned. The higher antioxidant potential of WOC can be linked to its enrichment in phenolic acids, particularly ellagic and gallic acids and their derivatives, which are well-documented for their potent antioxidant properties and lipid peroxidation inhibition (Badhani et al., 2015), WOC also presented a slightly higher amount of tocopherols.

Compared to the results herein reported, Grosso et al. (2018) also identified the oilcake obtained from Soxhlet defeated walnuts (variety Chandler) as a valuable source of polyphenols for walnut oil preservation, reporting IC_{50} values of 2.13 mg/mL and 2.57 mg/mL for water-soluble and ethyl acetate-soluble polyphenol extracts, respectively, in the DPPH assay. Santos et al. (2018) determined an IC_{50} of approximately 2 mg/mL for walnut oilcake extracts (variety Chandler) obtained by hydraulic pressing and subjected to various roasting conditions (50–150 °C), indicating that the antioxidant capacity is generally retained during roasting but may decrease when exposed to excessive heat. Values more similar to those obtained in the present work were reported by Burbano and Correa (2021), who observed an antioxidant response (IC_{50}) of 140 μg/mL in the DPPH assay for the extract obtained by maceration with acetone:water (70:30, v/v) from the oilcake produced by cold hydraulic pressing. Thus, our findings corroborate existing evidence that the oilcake preserves its antioxidant capacity after cold pressing extraction, which can be comparable to the antioxidant activity obtained from walnut kernels as demonstrated elsewhere (Pycia et al., 2020). Regarding to WOD, Gao et al. (2016) reported an IC_{50} of 1.48 mg/mL for the DPPH assay using an extract obtained by maceration with a 95% hydroethanolic solution. Discrepancies with the available literature may be related to the type of extraction method used, as well as intrinsic differences of samples, including edaphoclimatic and processing ones. Overall, the obtained results validate the antioxidant potential of the bio-residue of walnut oil production, which can be associated with the identified phenolic compounds (Table 2), suggesting that walnut by-products are a valuable source of antioxidant compounds.

3.3.2. Cytotoxicity and anti-inflammatory activity

The cytotoxicity assays, as summarized in Table 3, demonstrated that the oilcake extract exhibited no cytotoxic effects on the tested cell lines, including non-tumural cells, confirming its safety profile. In contrast, the WOD extract showed moderate cytotoxic activity against the tumor cell lines of human gastric epithelial (AGS) and human colorectal adenocarcinoma (Caco-2), with GI_{50} values of 234 to 254 μg/mL, respectively. The WOD extract also exhibited some toxicity against the PLP2 (porcine liver primary culture) cell line, however, showing a higher GI_{50} value compared to those for the AGS (gastric carcinoma) and Caco-2 (colorectal adenocarcinoma) cell lines, which suggests some preferential activity towards cancer cells.

Walnut by-products, such as green husks and leaves, have been previously studied for their cytotoxic potential. Ethanolic walnut leaf extracts demonstrated positive effects against HeLa cells (Vieira, Calhelha, et al., 2020), T47D-Kbluc (human breast cell line) and A549 (human lung adenocarcinoma) (Rusu et al., 2020). Additionally, walnut green husks showed significant cytotoxicity against MCF-7 (breast adenocarcinoma), NCI–H460 (non-small cell lung cancer), HeLa (cervical carcinoma), and HepG2 (hepatocellular carcinoma) (Vieira, Calhelha, et al., 2020). However, as far as the literature consulted, no data were available regarding the cytotoxicity of walnut oil industry by-products.

None of the walnut by-products exhibited anti-inflammatory activity in the nitric oxide inhibition assay, even at the highest concentration tested ($GI_{50} > 400$ μg/mL). To date, there is limited literature available on the anti-inflammatory properties of walnut oil processing by-products, emphasizing the need for further investigation.

3.3.3. Antimicrobial activity

The antibacterial activity results, summarized in Table 4, indicated that both WOC and WOD extracts showed limited antimicrobial activity against Gram-negative bacteria since for most assayed bacteria the MIC was >10 mg/mL. The WOC extract was able to inhibit *Y. enterocolitica* growth (MIC of 2.5 mg/mL), whereas WOD extract exhibited bacteriostatic activity against *E. cloacae*. No bactericidal effect (expressed as minimal bactericidal concentration, MBC) was observed against Gram-negative strains at the assayed concentration. Better inhibitory effects were observed against Gram-positive bacteria growth, particularly for *Staphylococcus aureus* (MIC = 1.25 mg/mL, WOD), *Listeria monocytogenes* (MIC = 2.5 mg/mL, WOC), and *Bacillus cereus* (MIC = 5 mg/mL, WOC). In addition, a bactericidal effect at 10 mg/mL was also observed against *S. aureus*.

The increased effectiveness of the extracts against Gram-positive bacteria aligns with previous findings and is likely attributed to the simpler structure of the Gram-positive cell wall, which lacks the

Table 3
Antioxidant and cytotoxicity activities of hydroethanolic extracts obtained from walnut oil by-products (μg/mL).

		WOC	WOD	E-223	E-302	Trolox	Ellipticine	Dexamethasone
Antioxidant activity (EC_{50})	TBARS	9.2 ± 0.2 ^{d}	33 ± 5 ^{b}	228 ± 10 ^{a}	284 ± 10 ^{a}	13.6 ± 0.1^{c}	–	–
	DPPH	130 ± 10 ^{b}	510 ± 17 ^{a}	43 ± 4 ^{c}	21 ± 3 ^{d}	42 ± 1^{c}	–	–
Cytotoxicity (IC_{50})	AGS	>400^{a}	254 ± 14^{a}	–	–	–	1.23 ± 0.03^{b}	–
	Caco-2	>400	234 ± 23^{a}	–	–	–	1.21 ± 0.02^{b}	–
	MCF-7	>400	>400	–	–	–	1.02 ± 0.02 ^{b}	–
	PLP2	>400	283 ± 3^{a}	–	–	–	1.40 ± 0.10^{b}	–
Anti-inflammatory activity (IC_{50})	RAW 264.7	>400	>400	–	–	–	–	6.3 ± 0.4

Data are expressed as mean ± SD (n = 3). WOD: walnut oil dregs. WOC: walnut oilcake. Positive controls: E-223 – sodium metabisulfite; E-302 – calcium ascorbate. Human gastric epithelial cell line (AGS), human colorectal adenocarcinoma (Caco-2), breast carcinoma (MCF-7) and primary cell line (PLP2). Different letters in the same row indicate significant differences ($p < 0.05$) between samples.

Table 4
Antimicrobial activity (mg/mL) of hydroethanolic extracts obtained from walnut oil by-products.

	WOC		WOD		Streptomicin		Methicilin		Ampicillin	
Gram-negative bacteria	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Enterobacter cloacae	10	>10	>10	>10	0.007	0.007	nt	nt	0.15	0.15
Escherichia coli	>10	>10	>10	>10	0.01	0.01	nt	nt	0.15	0.15
Pseudomonas aeruginosa	>10	>10	>10	>10	0.06	0.06	nt	nt	0.63	0.63
Salmonella enterocolitica	>10	>10	>10	>10	0.007	0.007	nt	nt	0.15	0.15
Yersinia enterocolitica	2.5	>10	>10	>10	0.007	0.007	nt	nt	0.15	0.15
Gram-positive bacteria										
Bacillus cereus	5	>10	10	>10	0.007	0.007	nt	nt	nt	nt
Listeria monocytogenes	2.5	>10	10	>10	0.007	0.007	nt	nt	0.15	0.15
Staphylococcus aureus	>10	>10	1.25	10	0.007	0.007	0.007	0.007	0.15	0.15

	WOC		WOD		Ketoconazole	
Fungi	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
Aspergillus brasiliensis	2.5	>10	>10	>10	0.06	0.125
Aspergillus fumigatus	5	>10	>10	>10	0.5	1

Data are mean ± SD ($n = 9$). Results are expressed as MIC (minimal inhibitory concentration), MBC (minimal bactericidal concentration), and MFC (minimal fungicidal concentration) in mg/mL freeze-dried extract). WOD: walnut oil dregs. WOC: walnut oilcake. nt: not-tested. Streptomicin, methicilin, ampicillin and ketoconazole served as positive controls at concentration of 1 mg/mL. nt: not-tested.

selective outer membrane characteristic of Gram-negative bacteria. The stronger activity against Gram-positive bacteria is consistent with the known ability of phenolic acids such as gallic and ellagic acids to disrupt bacterial membranes.While information on the antimicrobial properties of walnut oil processing by-products remains limited, studies on other walnut by-products, such as green husks and leaves, have demonstrated positive antimicrobial activity using hydroalcoholic extracts (Vieira, Pereira, et al., 2020).

Regarding antifungal activity, the WOC extract exhibited stronger inhibitory effects against *A. brasiliensis* (MIC of 2.5 mg/mL) and *A. fumigatus* (MIC of 5 mg/mL), two fungal strains commonly associated with food contamination. In contrast, the WOD extract showed no antifungal activity. These results may be related to the higher content of ellagitannins and glansrins of WOC. Neither WOC nor WOD extracts displayed fungicidal effects.

3.4. In vitro digestion and evaluation of the bioaccessibility of walnut polyphenols

To evaluate the stability of walnut by-products polyphenols throughout digestion, an *in vitro* gastrointestinal digestion model (INFOGEST protocol) was conducted and the composition of the bioaccessible fractions (BF), corresponding to the compounds that would be accessible to intestinal absorption after digestion, was determined (Table 5 and Fig. 1C and D).

The overall qualitative phenolic profile exhibited few changes after simulated digestion, with only two peaks, namely peak 16 present in the extracts of the starting materials and peak 8 found in WOC, not detected in bioaccessible fractions of either sample. Peak 16 corresponded to pentagalloyl glucose and valoneic acid dilactone, while peak 8 was identified as a mixture of glansreginic acid glycoside and glanrins. These compounds were likely hydrolysed into their simpler molecules under gastrointestinal conditions, explaining their absence in the bioaccessible fraction (Rocchetti et al., 2018). This hypothesis is supported by the levels of free glansreginic acid (peak 18), which presented one of the highest bioaccessibility values regardless of the sample (77% in WOC and 83% in WOD). In addition, glansreginin B, which can also form glansreginic acid *via* hydrolysis (Haramiishi et al., 2020), exhibited low levels in the bioaccessible fraction (only up to 7% and 11% in WOC and WOD, respectively), whereas glansreginin A, glansreginin C and glansreginic acid-*O*-hexoside demonstrated a similar low to moderate bioaccessibility following digestion (ranging from 14% to 40%, Table 5).

Similarly, the hydrolysable tannins pentagalloyl glucose and valoneic acid dilactone were most probably hydrolysed, generating gallic and ellagic acids. While ellagic acid was found to be the compound exhibiting the highest bioaccessibility (118% for WOC and 732% for WOD), gallic acid (peak 1), on the other hand, showed low relative bioaccessibility (12% and 40%, respectively). This may be due to the coelutions observed in peak 1, which hinders the precise quantification of this compound, therefore also of drawing precise conclusions on the digestive behaviour of compounds in peak 1, including gallic acid and its derivatives.

Besides the hydrolysable tannins of peaks 8 and 16, other ellagitannins also showed the lowest bioaccessibility values for both samples. For instance, only 1 to 2% and 10% of peaks 2 and 3 remained bioaccessible after digestion of WOC and WOD, respectively. The low relative abundance of these compounds in the bioaccessible fraction may be attributed to their chemical transformation during digestion, ultimately yielding ellagic acid as ellagitannins undergo ester bond hydrolysis. This is consistent with recent findings from simulated digestion of polyphenols from other walnut by-products, including walnut kernel pellicle rich in ellagitannins (Wang et al., 2024). As mentioned, ellagic acid (peak 13) was the most bioaccessible compound for either sample, with bioaccessible values surpassing 100%, which is highlighted by comparing Fig. 1C and D to Fig. 1A and B. In addition, tannins could have also interacted with other biomolecules during digestion to form insoluble compounds, further contributing to their low duodenal bioaccessibility (Engström et al., 2019).

Recent INFOGEST-focused literature highlights the influence of phenolic structural features on their digestive fate, with more complex or highly substituted phenolics being generally less stable or less soluble, while smaller, simpler phenolics often show higher bioaccessibility (Morales et al., 2025). Authors also reviewed several chemical and physicochemical phenomena occurring during gastrointestinal digestion that explain the variation in polyphenol composition upon this process. For instance, acidic and enzymatic hydrolysis or depolymerization of more complex molecules, oxidative reactions, or degradation of phenolic compounds, as well as precipitation of some compounds that became non-bioaccessible, and complexation with matrix or simulated fluid components (proteins including enzymes, carbohydrates, and

Table 5
Relative bioaccessibility (%) of polyphenols and related compounds of walnut oil processing by-products after *in vitro* digestion.

Peak	Rt (min)	Tentative identification	Bioaccessibility (%)	
			WOC	WOD
1a	4.01	Gallic acid	12 ± 0.1	40 ± 2
1b		1,2,3,4-Tetrahydro-2-oxo-4-quinolinecarboxylic acid 4-*O*-D-glucoside		
1c		HHDP-valoneoyl-glucose		
2a	4.22	Bis-HHDP-glucose	2 ± 0.1	10 ± 0.1
2b		Dihydrophaseic acid hexoside		
3a	4.52	Digalloyl-HHDP-glucose	1 ± 0.1	10 ± 0.1
3b		Ferulic acid-*O*-hexoside		
3c		ni		
4a	5.19	Brevifolin carboxylate	10 ± 0.1	30 ± 2
4b		HHDP-valoneoyl-glucose		
4c		Dihydroxy-coumarin derivative		
5a	6.32	Dicarboxylic acid derivative	15 ± 0.1	38 ± 1
5b		Ellagic acid-*O*-hexoside		
6a	6.6	*p*-Hydroxybenzoic acid	3 ± 0.1	14 ± 0.1
6b		Glansrin C		
6c		Digalloyl-HHDP-glucose		
6d		Benzyl-pentosylhexoside		
6e		Glansreginin B hexoside		
7a	7.23	Trigalloyl-glucose	17 ± 0.1	31 ± 2
7b		Roseoside		
8a	7.69	Glansreginic acid 8-*O*-β-D-glucoside + H_2O + 2H	nc	nc
8b		Glansrin C isomer		
8c		Glansrin A		
9a	8.21	Dihydrophaseic acid	6 ± 0.1	86 ± 2
9c		Galloyl-bis-HHDP-glucose		
10	10.19	Glansreginin B	7 ± 0.1	11 ± 1
11	10.60	Ellagic acid-*O*-pentoside	14 ± 0.1	183 ± 1
12a	10.91	Dicarboxylic acid derivative	14 ± 0.1	27 ± 1
12b		Procyanidin		
13	13.23	Ellagic acid	118 ± 1	732 ± 1
14	13.76	Glansreginic acid-*O*-hexoside	15 ± 1	34 ± 1
15	14.55	Glansreginin A hexoside	12 ± 0.1	42 ± 2
16a	15.00	Valoneic acid dilactone	nc	nc
16b		Penta-*O*-galloyl glucose		
17	17.60	Glansreginin A	15 ± 0.1	40 ± 0.1
18	17.96	Glansreginic acid	77 ± 2	83 ± 3
19	19.42	Glansreginin A isomer	30 ± 2	63 ± 3
20	20.84	Glansreginin C	14 ± 0.1	33 ± 1
21a	21.34	ni	25 ± 0.1	37 ± 1
21b		ni		
22	23.06	Glansreginin A–H_2O–2H	25 ± 0.1	36 ± 0.1
23	25.55	Dicarboxylic acid derivative	17 ± 0.1	41 ± 0.1
24	27.31	Glansreginin A-H_2O	15 ± 0.1	26 ± 0.1
25	27.74	Dicarboxylic acid derivative	14 ± 0.1	36 ± 0.1
		Average bioaccessibility	15 ± 0.1[a]	78 ± 0.1[b]

WOD: walnut oil dregs. WOC: walnut oilcake. Relative bioaccessibility refers to the fraction (%, mean ± SD) of extractable phenolic compounds from the samples (starting materials, WOC and WOD) that were solubilized in the respective aqueous, dialysable bioaccessible fractions upon completion of the duodenal phase. Average bioaccessibility considers the total polyphenol content (HPLC-DAD) before and after digestion. Peaks numbered according to the chromatogram shown in Table 2. Different letters in the same row indicate significant differences (*t* test, $p < 0.05$) between samples. ni: not identified.

ions), can modify the release, stability, and bioaccessibility of these compounds (Morales et al., 2025).

Although relative bioaccessibility values provide useful information about the retention of compounds after digestion, it is equally important to consider their absolute content, as it determines the actual amount available for uptake and absorption. Glansreginic acid showed a significant proportional increase in concentration between the non-digested samples and their bioaccessible fractions (exhibiting high relative bioaccessibility). However, its absolute quantity in both bioaccessible fractions was lower compared to compounds such as glansreginin A, which, despite having low relative bioaccessibility, were initially present in large amounts in the starting material (Table S1). In contrast, ellagic acid exhibited both the highest relative and absolute bioaccessibility, being the major compound in the BF of both samples.

Interestingly, on average, the relative bioaccessibility of total polyphenols was 78% after WOD digestion, while only 15% of WOC's polyphenols were bioaccessible (Table 5). In absolute terms, 1.17 ± 0.01 mg/g for WOD (fw) and 0.52 ± 0.01 mg/g for WOC (fw) were accessible for intestinal absorption upon completion of the duodenal phase (Table S1). This indicates that, despite the total extractable polyphenol content in WOC being nearly three times higher than in WOD, the overall (total) bioaccessibility of polyphenols was superior in WOD, underlining the importance of considering the balance between the initial concentration of compounds and their bioaccessibility in a particular sample. These results further support that the net bioaccessible polyphenols result from a complex interplay of multiple factors, considering not only the initial concentration and profile of compounds in the sample, but also the matrix composition, structure and processing level, in conjunction with the complexity of phenolic binding to its constituents, and following matrix release, the subsequent interactions, structural transformations and/or partial degradation these compounds may undergo, upon digestion conditions that include varied pH and enzyme activities, as mentioned above (Morales et al., 2025).

Literature shows that processing can lead to chemical or physical losses of sample compounds, but may also favour compound release, improving their extraction or bioaccessibility by modifying the sample matrix or compound structure. In this study, polyphenols from the WOD sample were revealed to be more bioaccessible than their counterparts

in WOC, which may be related to the by-product matrix, its composition and structural differences, and matrix–phenolic interactions impacting the final recovery of these compounds after digestion. WOC is a fiber- and protein-rich press-cake, with roughly 33% dietary fiber and 38% protein, being more structured than WOD, which consist of fine, oil-rich particles with much lower fiber and protein content. WOC's higher fiber content can interact with phenolic compounds or physically trap them, limiting their solubilisation in fluids during digestion or hindering enzymes' access (Morales et al., 2025). As a result, many phenolics can remain in the undigested colonic residue instead of the aqueous bioaccessible fraction. Moreover, many phenolic compounds are naturally bound to cell-wall structures, and unless digestion or processing breaks these associations, they remain in the non-bioaccessible residue (Núñez-Gómez et al., 2024). In addition, it is known that polyphenols can bind to proteins, potentially reducing their bioaccessibility (Mandalari et al., 2016). Since WOC has a higher protein content than WOD, it is possible that protein-polyphenol interactions may also have contributed to the lower bioaccessibility of polyphenols in WOC. In contrast, the less structured and lipid-rich matrix of WOD may help explain the higher bioaccessibility values observed for this fraction. Processing and reduced matrix particle size or rigidity can enhance polyphenol release. In matrices with higher lipid content, fat was associated with protection of some phenolics against degradation and losses by precipitation during digestion and favored micellarisation of more hydrophobic phenolic structures (Ortega et al., 2009).

It should be acknowledged that the conventional extraction approach used in this study primarily targeted the extractable phenolic fraction. Given the high fiber content of WOC, a substantial share of its phenolic compounds is likely present in a insoluble, bound or non-extractable form, associated with structural polysaccharides and lignocellulosic components of the cell wall (Núñez-Gómez et al., 2024). These non-extractable phenolics were not recovered and remained unquantified in the initial extracts. Such non-extractable phenolic compounds are generally less prone to be released during upper gastrointestinal digestion.

Although the bioaccessibility of several polyphenols from walnut by-products was relatively low, this does not preclude their contribution to health benefits. Both bound phenolic compounds and those retained in the colonic residue rather than solubilized into the aqueous bioaccessible fraction, *i.e.*, which are not bioaccessible in the upper gastrointestinal tract, can reach the colon, where they may be biotransformed by gut microbiota, potentially yielding bioactive metabolites that may be absorbed and exert systemic or local effects (Conte et al., 2023). This provides a plausible explanation for the pronounced prebiotic responses observed for the colonic residues, particularly from WOC, despite its comparatively low duodenal polyphenol bioaccessibility.

Human intervention studies have confirmed this route, particularly for ellagitannins present in walnuts, which are not absorbed and reach the colon where they are metabolized into urolithins by the gut microbiota. These metabolites have been detected in plasma, urine, and even human tissues, such as the prostate, following walnut or pomegranate intake, suggesting their bioavailability and potential role in disease prevention (García-Villalba et al., 2022). Therefore, even when duodenal bioaccessibility is limited, the colonic metabolism of these polyphenols remains a crucial pathway for their physiological effects.

3.5. *In vitro* bioavailability assay

The cellular uptake and basolateral transport of compounds of the bioaccessible fractions from *in vitro* digested samples was tested using a cell model of intestinal barrier, namely Caco-2 cells fully differentiated to enterocytes. Before carrying out the assay, the bioaccessible fractions were freeze-dried and diluted in DMEM to determine the non-toxic concentrations to be used in the bioavailability assay. TEER values were measured before and after the assay to monitor and guarantee the integrity of the cell monolayers.

After the transport assay has been carried out, the analysis by HPLC-DAD-ESI-MS/MS revealed a very low number of detectable compounds. This outcome can be largely attributed to the low concentrations of lyophilized bioaccessible fractions used (0.5 mg/mL for WOC and 0.25 mg/mL for WOD), which were pre-determined by the cytotoxicity screening to ensure monolayer viability. More specifically, we were able to identify only the major compound in both walnut by-products, namely glansreginin A, in the apical compartment. Nevertheless, glansreginin A, a dicarboxylic acid derivative, was absent in the basolateral fraction after the transport assay, which suggests poor membrane permeability and low bioavailability of this compound, yet basolateral concentrations may have remained below detection limits under the conditions. Although previous studies focusing on the bioavailability of this compound or data on its specific permeability are lacking, pharmacokinetic predictions indicate it has a very low epithelial permeability (log P_app ≈ -6.2) and high polar surface area ($\approx$230), characteristic of compounds with negligible transcellular transport (TCM-ADIP, 2025). These factors align with the observation that *walnut ellagitannins are generally unabsorbed in the small intestine.* Instead, their bioactivity *in vivo* is thought to arise after metabolism, as reviewed by Mateș et al. (2024); the authors further conclude that ellagitannins have "limited bioavailability" and do not reach high circulating levels on their own. Therefore, glansreginin A can be considered poorly permeable across the intestinal epithelium, which is consistent with its structure and size. As far as the literature consulted, this is the first attempt to evaluate the *in vitro* transport of phenolic compounds from walnut or walnut by-products in Caco-2 cells, therefore making it unfeasible the compare with previous results. Nevertheless, *in vivo* studies carried out in humans that consumed walnuts showed that the ingested ellagitannins and ellagic acid, including ellagic acid that becomes bioaccessible during digestion, mainly reach the colon where they are extensively metabolized by the gut flora to different compounds, mostly urolithins (Mateș et al., 2024; Pfundstein et al., 2014). In our study, although ellagic acid showed a high percentage of release during *in vitro* digestion, we could not detected it, possibly due to the low concentration of BF used in the assays, as mentioned, but also to matrix effects, background interference and limited solubility in cell culture media.

The limited intestinal transport observed for major walnut phenolics can be rationalised based on their structural characteristics. Compounds such as glansreginin A and hydrolysable tannins are characterized by moderate to high molecular weight and extensive hydroxylation. In Caco-2 models, passive transcellular diffusion is often limited by high polar surface area (Hou et al., 2004). In addition, Glansreginin A, being a dicarboxylic acid derivative with multiple hydroxyl groups, can be expected to be deprotonated, in an ionized state at physiological pH. This would increase its polarity and limit its passive transcellular permeation across the lipophilic enterocyte membrane (Neuhoff et al., 2005). Therefore, structural features typical of the major walnut compounds result in poor transepithelial transport. Consequently, such compounds are more likely to exert their biological effects indirectly, following microbial biotransformation in the colon rather than *via* direct systemic absorption. In the colon, unabsorbed ellagitannins serve as substrates for specific gut bacteria to produce urolithins, low-molecular-weight metabolites with proven anti-inflammatory and systemic bioavailability, as recently demonstrated in human clinical trials (Mateș et al., 2024; Moussa et al., 2025).

3.6. *Antioxidant capacity of bioaccessible fractions of polyphenols from walnut by-products*

In addition to the evaluation of the antioxidant activity of the hydroethanolic extracts prepared from both by-products, when performing the *in vitro* digestion, the antioxidant potential of the by-products was evaluated directly (Table 6). For this purpose, WOC and WOD samples were diluted in water at different concentrations using a

Table 6
Antioxidant activities of walnut by-products before and after *in vitro* digestion (mg/mL).

	TBARS		DPPH	
	Non-digested	Digested	Non-digested	Digested
WOC	2.6 ± 0.2[b]	0.4 ± 0.1 [b]	0.9 ± 0.1 [b]	0.4 ± 0.1 [a]
WOD	4.2 ± 0.6[a]	2.6 ± 0.8 [a]	2.5 ± 0.2 [a]	0.4 ± 0.1 [a]
p-value	0.0130	0.0077	0	1

Results are expressed as mean ± SD (n = 3). WOD: walnut oil dregs. WOC: walnut oilcake. Different letters in the same row indicate significant differences ($p < 0.05$) between samples.

vortex mixer to ensure proper dispersion and subsequently submitted to DPPH and TBARS assays. Similarly, following the INFOGEST protocol, the bioaccessible fraction (BF) was recovered, resuspended in water and subjected to antioxidant activity analysis. Interestingly, the BF of both by-products exhibited lower IC_{50} values compared to the undigested samples (Table 6), suggesting that the digestion process facilitated the release of bioactive compounds, such as phenolic molecules, which enhanced the samples' antioxidant potential.

These findings are consistent with previous research on the behaviour of polyphenols from nut by-products during gastrointestinal digestion, which also showed an increase in the antioxidant activity of the digestates. Duarte et al. (2023) demonstrated that *in vitro* digestion and colonic fermentation of almond bagasse significantly increased both phenolic content and antiradical capacity, while also modulating gut microbiota composition in a beneficial manner. Similarly, Herbello-Hermelo et al. (2018) reported high dialyzability ratios of total polyphenols in several nuts, including cashew and pistachio, emphasizing that the chemical structure of the compounds and the physical characteristics of the matrix influence bioavailability. In another study, Mandalari et al. (2010) demonstrated that almond skins released a significant fraction of polyphenols during digestion, highlighting the relevance of by-products as a source of bioaccessible antioxidants. Kamiloglu et al. (2014) further confirmed that nut components, particularly walnuts, retain substantial antioxidant capacity after gastrointestinal simulation, with synergistic effects observed when co-digested with dried fruits, suggesting that food matrix interactions can modulate polyphenol release and stability.

3.7. *Prebiotic potential of walnut by-products and their colonic residue after digestion*

The initial undigested walnut oil by-products (WOC and WOD) and the solid material obtained after *in vitro* digestion corresponding to the duodenal residues that would pass to the colon (here denoted as colon residues CR-WOD and CR-WOC), were subjected to prebiotic activity assessment using four colonic bacterial strains. Fructooligosaccharides (FOS) and glucose (GLU) were employed as positive controls. Fig. 2 illustrates the growth curve of three *Lactobacillus* and three *Bifidobacteria* strains by measuring turbidity (620 nm) and expressing it in optical density (OD) over time (intervals of 1 h for a total of 48 h). The turbidity generally exhibited an increase during the initial 20 h of the assay, thus indicating an increasing of microorganism biomass. The maximum observed O.D. along with the respective time in hours are presented in Table S1.

In general, both the undigested (WOC, WOD) and the non-bioaccessible fractions (CR-WOC, CR-WOD) of walnut by-products demonstrated the ability to stimulate the growth of the tested bacteria. However, differences were observed across strains regarding bacteria growth, substrate utilization and fermentation dynamics, particularly between lactobacilli and bifidobacteria. Among lactobacilli, CR-WOD and WOC exhibited the highest prebiotic activity after 48 h, as evidenced by enhanced growth of *L. rhamnosus* and L. *casei* compared to

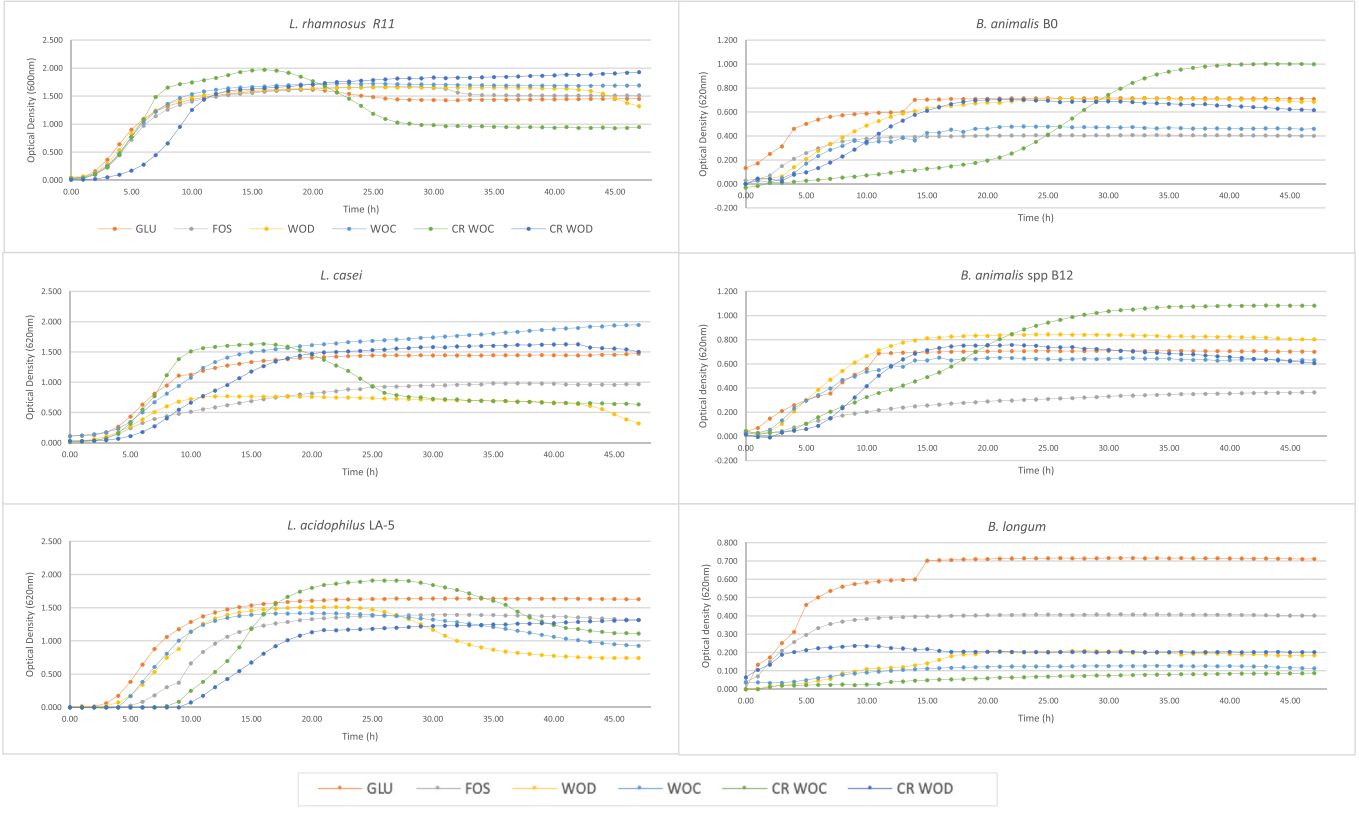

Fig. 2. Growth curves of *Bifidobacterium* and *Lactobacillus* probiotic strains in medium containing glucose (GLU), fructooligosaccharides (FOS), walnut oil dregs (WOD), colonic residue of WOD (CR-WOD), walnut oilcake (WOC), and colonic residue of oilcake (CR-WOC) at 20 mg/mL (2% *w/v*) during 48 h.

the positive controls (FOS and glucose), suggesting sustained metabolic activity. Overall, the WOD sample showed an increased prebiotic activity after *in vitro* digestion. Although the non-bioaccessible fraction obtained after *in vitro* digestion (CR-WOD) showed a longer lag phase in the three lactobacilli strains, it consistently resulted in higher (*L. rhamnosus* and *L. casei*) or similar (*L. acidophilus* LA-5) growth levels compared to FOS, a widely used commercial prebiotic, thus indicating its strong potential as a prebiotic. Regarding the oilcake samples, CR-WOC also showed interesting results since it attained the highest O.D. values for both *L. rhamnosus* R11 and *L. acidophilus* LA-5. In *L. rhamnosus* R11 and *L. casei,* CR-WOC led to a rapid initial increase in optical density, suggesting a fast fermentation of accessible components. However, this was followed by a marked decline in growth after approximately 18 h, likely due to excessive acidification resulting from rapid fermentation or depletion of readily available nutrients. A similar trend was observed in *L. acidophilus* LA-5, although this strain exhibited a longer lag phase, and the decline in O.D. occurred later, around 30 h.

Among the bifidobacteria tested, CR-WOC exhibited the strongest prebiotic effect for *B. animalis* Bo and B12, achieving the highest final O.D. after 48 h. Although CR-WOC showed a delayed onset of growth compared to glucose and FOS, its sustained increase in O.D. suggests a gradual and prolonged fermentation. In both strains, digestion improved the prebiotic effect of walnut oil cake, as CR-WOC led to higher bacterial growth than the undigested WOC. Regarding walnut dregs, although digestion did not improve the prebiotic effect of WOD, both CR-WOD and WOD still supported better growth than FOS, evidencing that walnut dregs may still be more effective than this common prebiotic for these strains. Finally, *B. longum* showed poor growth across all walnut-derived samples and moderate growth for FOS, with only glucose leading to a meaningful increase in O.D., which suggests a narrower carbohydrate fermentation profile of this strain.

The prebiotic potential observed for both undigested and digested walnut by-products aligns with previous findings that highlight the capacity of walnuts to modulate gut microbiota and stimulate the growth of beneficial strains. In a human clinical trial, Bamberger et al. (2018) demonstrated that daily walnut consumption significantly increased the abundance of *Bifidobacterium spp.* and *Ruminococcaceae*, supporting a bifidogenic effect. Similarly, Wang et al. (2021) reported that soluble fiber from walnut meals enhanced the production of short-chain fatty acids and restored microbial balance in mice models. In the present study, the enhanced growth of *Lactobacillus* and *Bifidobacterium* strains particularly when testing the non-bioaccessible fractions (CR-WOD, CR-WOC), reflects the gradual fermentation profile described for slowly fermentable dietary fibers. Overall, the results demonstrated that by-products from the walnut oil industry can serve as a source of beneficial compounds, such as fibers and fermentable carbohydrates, promoting the growth of different probiotic strains, potentially enhancing gastrointestinal functions.

The antioxidant and other activities measured after simulated digestion reflect the fraction of phenolics that becomes bioaccessible and the qualitative changes in their profile following digestion. As aforementioned, phenolic can undergo several structural modifications under gastrointestinal conditions, thereby altering their stability, reactivity and redox behaviour.

In our case, although WOC showed higher polyphenol content before digestion, its lower duodenal bioaccessibility likely constrained the antioxidant effectiveness of its bioaccessible fraction relative to WOD, supporting that total phenolics in the starting material are not sufficient predictors of activity. This interpretation is consistent with recent evidence that digestion can increase, decrease, or yield mixed bioactivity outcomes depending on matrix effects and transformation pathways, and that correlations between specific phenolics (and derived products) and bioactivity remain necessary (Morales et al., 2025). This context also helps to explain why bioaccessible fractions can exhibit higher antioxidant activity after digestion despite a lower total phenolic content than their respective starting materials, since digestion can generate smaller, more reactive aglycones from larger molecules. For instance, digestion may promote the hydrolysis of glycosides and tannins into aglycones (*e.g.*, ellagic acid) that often exhibit superior radical-scavenging efficiency due to the unmasking of hydroxyl groups previously involved in ester or glycosidic bonds (Wang et al., 2024).

In contrast to WOC, WOD exhibited higher post-digestion bioaccessibility and maintained antioxidant, antimicrobial and cytotoxic activities, supporting a stronger relationship between the bioaccessible phenolic fraction and biological effects. Therefore, overall, the bioactivities of WOC and WOD correlate with their phenolic profiles post-digestion: WOD's higher content of bioaccessible phenolics corresponded to stronger activities in the soluble fraction, whereas WOC's non-bioaccessible polyphenols and bound polyphenols were associated with high prebiotic effects. These indirect effects manifested later in the colon may result in health outcomes *via* gut microbiota modulation.

4. Conclusion

The chemical analysis of walnut oilcake (WOC) and dregs (WOD) revealed that WOD had higher levels of fat and available carbohydrates, while the oilcake was rich in protein, fibers, and organic acids. Both by-products showed a similar fatty acid profile with a prevalence of PUFA and both presented high levels of vitamin E. The qualitative phenolic profile was also similar between the samples, with glansreginin A being the major compound in both samples, though with minor differences due to the absence of minor compounds such as glansrin A in the dregs. Quantitatively, WOC was shown to be a richer source of bioactive phenolic compounds than WOD. However, after *in vitro* digestion, in general, compounds were found to be more bioaccessible in WOD compared to WOC. Due to cytotoxic effects, a very low concentration of the bioaccessible fractions was tested in the transport assay across Caco-2 cells, which may explain the very low number of compounds detected in the different compartments. More specifically, only the major compound in both samples, glansreginin A, was detected in the apical compartment, indicating a lack of transport across the membrane and suggesting that this compound is not bioavailable.

Both by-products had high antioxidant potential, as demonstrated by their lower IC_{50} values in lipid peroxidation inhibition (TBARS) and DPPH assays compared to commercial antioxidants. Moreover, both WOD and WOC showed higher antioxidant activity after *in vitro* digestion, which may be related to the increase of some bioactive compounds in the bioaccessible fraction. This sustained antioxidant effect suggests a protective role in the gut environment, potentially contributing to the mitigation of oxidative stress in the colon.

Moreover, this study demonstrates that, after digestion, the non-bioaccessible fractions of the walnut oil industry by-products may reach the colon and act as a fermentable substrate for beneficial gut microbiota. The results demonstrate that walnut oil by-products support the growth and metabolic activity of probiotic bacteria, in some cases surpassing conventional prebiotics such as fructooligosaccharides. Overall, the results show that walnut oil cake, particularly after digestion (CR-WOC), has strong prebiotic potential, especially for *B. animalis* and some *Lactobacillus* strains. The observed differences in fermentation patterns for this sample, *i.e.*, rapid but short growth in some *Lactobacillus* strains and slower growth in some *Bifidobacterium* strains suggest that this substrate may offer complementary effects, potentially supporting distinct bacteria across different regions of the colon, contributing to gut health improvement.

Beyond valorisation, the potential incorporation of WOD and WOC into food products presents promising opportunities for functional food development. These by-products could be used as partial substitutes in bakery goods, snacks, or beverages, contributing additional fiber, phenolics, and other bioactive compounds. Nevertheless, careful attention to potential sensory impacts should be considered, with formulation strategies aimed at maintaining product acceptability.

Further research is still needed to confirm the potential health

benefits and applications of these by-products. Nevertheless, the current results highlight their value and offer useful insights for developing new food products and functional ingredients.

CRediT authorship contribution statement

Rafael Mascoloti Spréa: Writing – original draft, Methodology, Investigation, Conceptualization. **Daniele Bobrowski Rodrigues:** Writing – original draft, Methodology, Investigation. **Tânia C.S. Pires:** Methodology. **Ricardo C. Calhelha:** Methodology. **María E. Brassesco:** Writing – review & editing, Methodology, Investigation. **Manuela Pintado:** Resources, Investigation. **Susana Santoyo:** Resources, Investigation. **Elena Arranz:** Writing – review & editing, Methodology, Investigation. **Miguel A. Prieto:** Writing – review & editing, Supervision. **Lillian Barros:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Joana S. Amaral:** Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2026.149641.

Data availability

Data will be made available on request.

References

AOAC. (2016). *Official methods of analysis of AOAC international*. Rockvile, MD, USA: AOAC International.

Arranz, E., Santoyo, S., Jaime, L., Fornari, T., Reglero, G., Guri, A., & Corredig, M. (2015). Improved bioavailability of supercritical rosemary extract through encapsulation in different delivery systems after in vitro digestion. *Food Digestion Research and Current Opinion, 6*, 30–37. https://doi.org/10.1007/s13228-015-0040-9

Badhani, B., Sharma, N., & Kakkar, R. (2015). Gallic acid: A versatile antioxidant with promising therapeutic and industrial applications. *RSC Advances, 5*, 27540–27557. https://doi.org/10.1039/C5RA01911G

Bakkalbasi, E. (2019). Oxidative stability of enriched walnut oil with phenolic extracts from walnut press-cake under accelerated oxidation conditions and the effect of ultrasound treatment. *Journal of Food Measurement and Characterization, 13*, 43–50. https://doi.org/10.1007/s11694-018-9917-y

Bakkalbasi, E., Meral, R., & Dogan, I. S. (2015). Bioactive compounds, physical and sensory properties of cake made with walnut press-cake. *Journal of Food Quality, 38*, 422–430. https://doi.org/10.1111/jfq.12169

Bamberger, C., Rossmeier, A., Lechner, K., Wu, L., Waldmann, E., Fischer, S., … Parhofer, K. G. (2018). A walnut-enriched diet affects gut microbiome in healthy Caucasian subjects: A randomized, controlled trial. *Nutrients, 10*, Article 244. https://doi.org/10.3390/nu10020244

Barros, L., Carvalho, A. M., Morais, J. S., & Ferreira, I. C. F. R. (2010). Strawberry-tree, blackthorn and rose fruits: Detailed characterisation in nutrients and phytochemicals with antioxidant properties. *Food Chemistry, 120*, 247–254. https://doi.org/10.1016/j.foodchem.2009.10.016

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., … Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols, 14*, 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Burbano, J. J., Cabezas, D. M., & Correa, M. J. (2022). Gluten-free cakes with walnut flour: A technological, sensory, and microstructural approach. *International Journal of Food Science & Technology, 57*, 4772–4781. https://doi.org/10.1111/ijfs.15591

Burbano, J. J., & Correa, M. J. (2021). Composition and physicochemical characterization of walnut flour, a by-product of oil extraction. *Plant Foods for Human Nutrition, 76*, 233–239. https://doi.org/10.1007/s11130-021-00898-4

Clinical and Laboratory Standards Institute. (2018). *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically*.

Conte, A., Martini, S., & Tagliazucchi, D. (2023). Influence of processing and digestion on the stability, bioac-Cessibility and bioactivity of food polyphenols. *Foods, 12*, 851. https://doi.org/10.3390/foods12040851

D'Antuono, I., Garbetta, A., Linsalata, V., Minervini, F., & Cardinali, A. (2015). Polyphenols from artichoke heads (Cynara cardunculus (L.) subsp. scolymus Hayek): In vitro bio-accessibility, intestinal uptake and bioavailability. *Food & Function, 6*, 1268–1277. https://doi.org/10.1039/C5FO00137D

Duarte, H., Carrera, C., Aliaño-González, M. J., Gutiérrez-Escobar, R., Jiménez-Hierro, M. J., Palma, M., … Medronho, B. (2023). On the valorization of *Arbutus unedo* L. pomace: Polyphenol extraction and development of novel functional cookies. *Foods, 12*, Article 3707. https://doi.org/10.3390/foods12193707

Engström, M. T., Arvola, J., Nenonen, S., Virtanen, V. T. J., Leppä, M. M., Tähtinen, P., & Salminen, J.-P. (2019). Structural features of Hydrolyzable tannins determine their ability to form insoluble complexes with bovine serum albumin. *Journal of Agricultural and Food Chemistry, 67*, 6798–6808. https://doi.org/10.1021/acs.jafc.9b02188

de la Fuente, B., Pinela, J., Mandim, F., Heleno, S. A., Ferreira, I. C. F. R., Barba, F. J., Berrada, H., Caleja, C., & Barros, L. (2022). Nutritional and bioactive oils from salmon (Salmo salar) side streams obtained by Soxhlet and optimized microwave-assisted extraction. *Food Chemistry, 386*, Article 132778. https://doi.org/10.1016/j.foodchem.2022.132778

Gao, L., Kaize, H.E., Yanqiang, D., Qiang P.U, WANG S., 2016. The antioxidant and glycosidase inhibitory activities of walnut dreg extracts. Chinese Journal of Applied & Environmental Biology 64–69. doi:https://doi.org/10.3724/SP.J.1145.2015.03035.

Gao, P., Liu, R., Jin, Q., & Wang, X. (2019). Comparison of solvents for extraction of walnut oils: Lipid yield, lipid compositions, minor-component content, and antioxidant capacity. *LWT, 110*, 346–352. https://doi.org/10.1016/j.lwt.2019.04.100

García-Villalba, R., Giménez-Bastida, J. A., Cortés-Martín, A., Ávila-Gálvez, M.Á., Tomás-Barberán, F. A., Selma, M. V., … González-Sarrías, A. (2022). Urolithins: A comprehensive update on their metabolism, bioactivity, and associated gut microbiota. *Molecular Nutrition & Food Research, 66*, Article 2101019. https://doi.org/10.1002/mnfr.202101019

Gómez-García, R., Vilas-Boas, A. A., Oliveira, A., Amorim, M., Teixeira, J. A., Pastrana, L., … Campos, D. A. (2022). Impact of simulated human gastrointestinal digestion on the bioactive fraction of upcycled pineapple by-products. *Foods, 11*, 126. https://doi.org/10.3390/foods11010126

Grosso, A. L., Asensio, C. M., Nepote, V., & Grosso, N. R. (2018). Antioxidant activity displayed by phenolic compounds obtained from walnut oil cake used for walnut oil preservation. *Journal of the American Oil Chemists' Society, 95*, 1409–1419. https://doi.org/10.1002/aocs.12145

Grosso, A. L., Riveros, C., Asensio, C. M., Grosso, N. R., & Nepote, V. (2020). Improving walnuts' preservation by using walnut phenolic extracts as natural antioxidants through a walnut protein-based edible coating. *Journal of Food Science, 85*, 3043–3051. https://doi.org/10.1111/1750-3841.15395

Haramiishi, R., Okuyama, S., Yoshimura, M., Nakajima, M., Furukawa, Y., Ito, H., & Amakura, Y. (2020). Identification of the characteristic components in walnut and anti-inflammatory effect of glansreginin a as an indicator for quality evaluation. *Bioscience, Biotechnology, and Biochemistry, 84*, 187–197. https://doi.org/10.1080/09168451.2019.1670046

Herbello-Hermelo, P., Lamas, J. P., Lores, M., Domínguez-González, R., Bermejo-Barrera, P., & Moreda-Piñeiro, A. (2018). Polyphenol bioavailability in nuts and seeds by an in vitro dialyzability approach. *Food Chemistry, 254*, 20–25. https://doi.org/10.1016/j.foodchem.2018.01.183

Hong, Z., Xie, J., Tao, L., Dai, J.-J., Li, T., Zhang, L., Bai, Y., Hu, X., Chen, J., Sheng, J., & Tian, Y. (2024). Exploration of cyclooxygenase-2 inhibitory peptides from walnut dreg proteins based on in silico and in vitro analysis. *Food Science and Human Wellness, 13*, 1636–1644. https://doi.org/10.26599/FSHW.2022.9250143

Hou, T. J., Zhang, W., Xia, K., Qiao, X. B., & Xu, X. J. (2004). ADME evaluation in drug discovery. 5. Correlation of Caco-2 permeation with simple molecular properties. *Journal of Chemical Information and Computer Sciences, 44*, 1585–1600. https://doi.org/10.1021/ci049884m

Ito, H., Okuda, T., Fukuda, T., Hatano, T., & Yoshida, T. (2007). Two novel dicarboxylic acid derivatives and a new dimeric Hydrolyzable tannin from walnuts. *Journal of Agricultural and Food Chemistry, 55*, 672–679. https://doi.org/10.1021/jf062872b

Jia, X., Luo, H., Xu, M., Zhai, M., Guo, Z., Qiao, Y., & Wang, L. (2018). Dynamic changes in Phenolics and antioxidant capacity during pecan (Carya illinoinensis) kernel ripening and its Phenolics profiles. *Molecules, 23*, 435. https://doi.org/10.3390/molecules23020435

Kamiloglu, S., Pasli, A. A., Ozcelik, B., & Capanoglu, E. (2014). Evaluating the in vitro bioaccessibility of phenolics and antioxidant activity during consumption of dried fruits with nuts. *LWT - Food Science and Technology, 56*, 284–289. https://doi.org/10.1016/j.lwt.2013.11.040

Kuete, V., Ango, P. Y., Fotso, G. W., Kapche, G. D., Dzoyem, J. P., Wouking, A. G., … Abegaz, B. M. (2011). Antimicrobial activities of the methanol extract and compounds from Artocarpus communis (Moraceae). *BMC Complementary and Alternative Medicine, 11*, 42. https://doi.org/10.1186/1472-6882-11-42

Liu, D., Di, H., Guo, Y., Betchem, G., & Ma, H. (2022). Multi-mode S-type ultrasound-assisted protein extraction from walnut dregs and in situ real-time process monitoring. *Ultrasonics Sonochemistry, 89*, Article 106116. https://doi.org/10.1016/j.ultsonch.2022.106116

Mandalari, G., Tomaino, A., Rich, G. T., Lo Curto, R., Arcoraci, T., Martorana, M., Bisignano, C., Saija, A., Parker, M. L., Waldron, K. W., & Wickham, M. S. J. (2010). Polyphenol and nutrient release from skin of almonds during simulated human digestion. *Food Chemistry, 122*, 1083–1088. https://doi.org/10.1016/j.foodchem.2010.03.079

Mandalari, G., Vardakou, M., Faulks, R., Bisignano, C., Martorana, M., Smeriglio, A., & Trombetta, D. (2016). Food matrix effects of polyphenol bioaccessibility from almond skin during simulated human digestion. *Nutrients, 8*, 568. https://doi.org/10.3390/nu8090568

Mandim, F., Barros, L., Calhelha, R. C., Abreu, R. M. V., Pinela, J., Alves, M. J., Heleno, S., Santos, P. F., & Ferreira, I. C. F. R. (2019). Calluna vulgaris (L.) Hull: Chemical characterization, evaluation of its bioactive properties and effect on the vaginal microbiota. *Food and Function, 10*, 78–89. https://doi.org/10.1039/c8fo01910j

Mateș, L., Banc, R., Zaharie, F. A., Rusu, M. E., & Popa, D.-S. (2024). Mechanistic insights into the biological effects and antioxidant activity of walnut (*Juglans regia* L.) ellagitannins: A systematic review. *Antioxidants, 13*, Article 974. https://doi.org/10.3390/antiox13080974

Medic, A., Jakopic, J., Hudina, M., Solar, A., & Veberic, R. (2021). Identification and quantification of the major phenolic constituents in Juglans regia L. peeled kernels and pellicles, using HPLC–MS/MS. *Food Chemistry, 352*, Article 129404. https://doi.org/10.1016/j.foodchem.2021.129404

Moilanen, J., Sinkkonen, J., & Salminen, J.-P. (2013). Characterization of bioactive plant ellagitannins by chromatographic, spectroscopic and mass spectrometric methods. *Chemoecology, 23*, 165–179. https://doi.org/10.1007/s00049-013-0132-3

Morales, D., Iriondo-DeHond, A., & Fernández-Tomé, S. (2025). Application of the INFOGEST 2.0 standardized method to study the behavior of phenolic compounds throughout gastrointestinal digestion. *Food Chemistry, 492*, Article 145531. https://doi.org/10.1016/j.foodchem.2025.145531

Moussa, M. R., Fan, N., Birk, J., Provatas, A. A., Mehta, P., Hatano, Y., … Rosenberg, D. W. (2025). Systemic inflammation and the inflammatory context of the colonic microenvironment are improved by Urolithin a. *Cancer Prevention Research (Philadelphia, Pa.), 18*, 235–250. https://doi.org/10.1158/1940-6207.CAPR-24-0383

Neuhoff, S., Ungell, A.-L., Zamora, I., & Artursson, P. (2005). pH-dependent passive and active transport of acidic drugs across Caco-2 cell monolayers. *European Journal of Pharmaceutical Sciences, 25*, 211–220. https://doi.org/10.1016/j.ejps.2005.02.009

Núñez-Gómez, V., Jesús Periago, M., Luis Ordóñez-Díaz, J., Pereira-Caro, G., Manuel Moreno-Rojas, J., & González-Barrio, R. (2024). Dietary fibre fractions rich in (poly) phenols from orange by-products and their metabolisation by in vitro digestion and colonic fermentation. *Food Research International, 177*, Article 113718. https://doi.org/10.1016/j.foodres.2023.113718

Orsavova, J., Misurcova, L., Ambrozova, J. V., Vicha, R., & Mlcek, J. (2015). Fatty acids composition of vegetable oils and its contribution to dietary energy intake and dependence of cardiovascular mortality on dietary intake of fatty acids. *International Journal of Molecular Sciences, 16*, 12871–12890. https://doi.org/10.3390/ijms160612871

Ortega, N., Reguant, J., Romero, M.-P., Macià, A., & Motilva, M.-J. (2009). Effect of fat content on the digestibility and bioaccessibility of cocoa polyphenol by an in vitro digestion model. *Journal of Agricultural and Food Chemistry, 57*, 5743–5749. https://doi.org/10.1021/jf900591q

Pascoalino, L. A., Pires, T. C., Pinela, J., Rodrigues, M.Â., Ferreira, I. C., Barros, L., Barreira, J. C., & Reis, F. S. (2025). Foliar application of biostimulants improves nutritional and bioactive quality of walnuts. *Journal of the Science of Food and Agriculture, 105*, 1138–1146. https://doi.org/10.1002/jsfa.13904

Pfundstein, B., Haubner, R., Würtele, G., Gehres, N., Ulrich, C. M., & Owen, R. W. (2014). Pilot walnut intervention study of urolithin bioavailability in human volunteers. *Journal of Agricultural and Food Chemistry, 62*, 10264–10273. https://doi.org/10.1021/jf5040652

Pop, A., Păucean, A., Socaci, S. A., Alexa, E., Man, S. M., Mureșan, V., … Muste, S. (2020). Quality characteristics and volatile profile of macarons modified with walnut oilcake by-product. *Molecules, 25*, 2214. https://doi.org/10.3390/molecules25092214

Publications Office of the European Union. (2011). *Regulation (EU) no 1169/2011 of the European Parliament and of the council.* . (Official Journal of the European Union).

Pycia, K., Kapusta, I., & Jaworska, G. (2019). Impact of the degree of maturity of walnuts (Juglans regia L.) and their variety on the antioxidant potential and the content of tocopherols and polyphenols. *Molecules, 24*, 2936. https://doi.org/10.3390/molecules24162936

Pycia, K., Kapusta, I., & Jaworska, G. (2020). Walnut oil and oilcake affect selected the physicochemical and antioxidant properties of wheat bread enriched with them. *Journal of Food Processing and Preservation, 44*. https://doi.org/10.1111/jfpp.14573

Rabadán, A., Pardo, J. E., Gómez, R., & Álvarez-Ortí, M. (2018). Evaluation of physical parameters of walnut and walnut products obtained by cold pressing. *LWT, 91*, 308–314. https://doi.org/10.1016/j.lwt.2018.01.061

Regueiro, J., Sánchez-González, C., Vallverdú-Queralt, A., Simal-Gándara, J., Lamuela-Raventós, R., & Izquierdo-Pulido, M. (2014). Comprehensive identification of walnut polyphenols by liquid chromatography coupled to linear ion trap–Orbitrap mass spectrometry. *Food Chemistry, 152*, 340–348. https://doi.org/10.1016/j.foodchem.2013.11.158

Ren, S., Zhang, Q., Jiang, M., Chen, M., Xu, X., Wang, D., Pan, Y., & Liu, X. (2022). Systematic characterization of the metabolites of defatted walnut powder extract in vivo and screening of the mechanisms against NAFLD by UPLC-Q-Exactive Orbitrap MS combined with network pharmacology. *Journal of Ethnopharmacology, 285*, Article 114870. https://doi.org/10.1016/j.jep.2021.114870

Rocchetti, G., Chiodelli, G., Giuberti, G., & Lucini, L. (2018). Bioaccessibility of phenolic compounds following in vitro large intestine fermentation of nuts for human consumption. *Food Chemistry, 245*, 633–640. https://doi.org/10.1016/j.foodchem.2017.10.146

Rodrigues, D. B., Veríssimo, L., Finimundy, T., Rodrigues, J., Oliveira, I., Gonçalves, J., … Calhelha, R. C. (2023). Chemical and bioactive screening of green polyphenol-Rich extracts from chestnut by-products: An approach to guide the sustainable production of high-added value ingredients. *Foods, 12*, 2596. https://doi.org/10.3390/foods12132596

Rusu, M. E., Fizesan, I., Pop, A., Mocan, A., Gheldiu, A.-M., Babota, M., … Popa, D.-S. (2020). Walnut (Juglans regia L.) septum: Assessment of bioactive molecules and in vitro biological effects. *Molecules, 25*, 2187. https://doi.org/10.3390/molecules25092187

Santos, J., Alvarez-Ortí, M., Sena-Moreno, E., Rabadán, A., Pardo, J.E., Beatriz PP Oliveira, M., 2018. Effect of roasting conditions on the composition and antioxidant properties of defatted walnut flour. Journal of the Science of Food and Agriculture 98, 1813–1820. doi:https://doi.org/10.1002/jsfa.8657.

Slatnar, A., Mikulic-Petkovsek, M., Stampar, F., Veberic, R., & Solar, A. (2015). Identification and quantification of phenolic compounds in kernels, oil and bagasse pellets of common walnut (Juglans regia L.). *Food Research International, 67*, 255–263. https://doi.org/10.1016/j.foodres.2014.11.016

Song, H., Cong, Z., Wang, C., He, M., Liu, C., & Gao, P. (2022). Research progress on walnut oil: Bioactive compounds, health benefits, extraction methods, and medicinal uses. *Journal of Food Biochemistry, 46*, Article e14504. https://doi.org/10.1111/jfbc.14504

Spréa, R. M., Finimundy, T. C., Calhelha, R. C., Pires, T. C. S. P., Prieto, M. A., Amaral, J. S., & Barros, L. (2024). Comprehensive analysis of soybean (*Glycine max* L.) by-products: Nutritional value, phenolic composition, and bioactive properties. *Food Bioscience, 62*, Article 105382. https://doi.org/10.1016/j.fbio.2024.105382

Tang, L., Hu, M., Bai, S., Wang, B., Fan, B., Zhang, L., & Wang, F. (2024). Extraction of insoluble soybean fiber by alternating ultrasonic/alkali and its improved superior physicochemical and functional properties. *International Journal of Biological Macromolecules, 263*, Article 130505. https://doi.org/10.1016/j.ijbiomac.2024.130505

TCM-ADIP. (2025). *Glansreginin A – ADME profile and pharmacokinetic predictions*.

Ungar, Y., Osundahunsi, O. F., & Shimoni, E. (2003). Thermal stability of Genistein and Daidzein and its effect on their antioxidant activity. *Journal of Agricultural and Food Chemistry, 51*, 4394–4399. https://doi.org/10.1021/jf034021z

Vieira, V., Calhelha, R.C., Barros, L., Coutinho, J.A.P., C. F. R. Ferreira, I., Ferreira, O., 2020. Insights on the extraction performance of alkanediols and glycerol: Using *Juglans regia* L. leaves as a source of bioactive compounds. Molecules 25, 2497. doi: https://doi.org/10.3390/molecules25112497.

Vieira, V., Pereira, C., Abreu, R. M. V., Calhelha, R. C., Alves, M. J., Coutinho, J. A. P., Ferreira, O., Barros, L., & Ferreira, I. C. F. R. (2020). Hydroethanolic extract of Juglans regia L. green husks: A source of bioactive phytochemicals. *Food and Chemical Toxicology, 137*, Article 111189. https://doi.org/10.1016/j.fct.2020.111189

Villalva, M., Jaime, L., Siles-Sánchez, M.d.l. N., & Santoyo, S. (2022). Bioavailability assessment of yarrow phenolic compounds using an in vitro digestion/Caco-2 cell model: Anti-inflammatory activity of basolateral fraction. *Molecules, 27*, Article 8254. https://doi.org/10.3390/molecules27238254

Wang, G., Zhong, D., Liu, H., Yang, T., Liang, Q., Wang, J., Zhang, R., & Zhang, Y. (2021). Water soluble dietary fiber from walnut meal as a prebiotic in preventing metabolic syndrome. *Journal of Functional Foods, 78*, Article 104358. https://doi.org/10.1016/j.jff.2021.104358

Wang, Y., Cao, S., Meng, Y., Cheng, Y., Han, Z., & Wang, F. (2024). Mechanisms underlying the effect of walnut pellicle extracts and its four representative polyphenols on in vitro digestion of walnut protein isolate. *Food and Bioproducts Processing, 144*, 166–177. https://doi.org/10.1016/j.fbp.2024.01.013

Xu, S., Ou, C., Chen, Y., Xiao, H., Fu, C., Lu, W., & Wu, Z. (2020). Study on the pyrolysis characteristics of walnut processing by-products. *IOP Conference Series: Earth and Environmental Science, 508*, Article 012126. https://doi.org/10.1088/1755-1315/508/1/012126